Effects of the Steroid Antagonist RU486 on Dimerization of the Human Progesterone Receptor†

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ABSTRACT: We previously reported, using a coimmunoprecipitation assay, that the B form (PR-B) of the human progesterone receptor from T47D human breast cancer cells dimerizes in solution with the A receptor (PR-A) and that the extent of dimerization correlates with receptor binding activity for specific DNA sequences [DeMarzo, A. M., Beck, C. A., Oñate, S. A., & Edwards, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 72–76]. This suggested that solution dimerization is an intermediate step in the receptor activation process. The present study has tested the effects of the progesterone antagonist RU486 on solution dimerization of progesterone receptors (PR). As determined by the coimmunoprecipitation assay, RU486 binding did not impair dimerization of receptors; rather, the antagonist promoted more efficient solution dimerization than the progestin agonist R5020. This enhanced receptor dimerization correlated with a higher DNA binding activity for transformed receptors bound with RU486. RU486 has been shown previously to produce two other alterations in the human PR when compared with R5020. PR–RU486 complexes in solution exhibit a faster sedimentation rate (6 S) on salt-containing sucrose density gradients than PR–R5020 complexes (4 S), and PR–DNA complexes have a faster electrophoretic mobility on gel-shift assays in the presence of RU486. We presently show that the 6 S PR–RU486 complex is a receptor monomer, not a dimer. The increased sedimentation rate and increased mobility on gel-shift assays promoted by RU486 were also observed with recombinant PR-A and PR-B separately expressed in insect cells from baculovirus vectors. These results suggest that RU486 induces a distinct conformational change both in PR monomers in solution and in dimers bound to DNA. We also examined whether conformational changes in PR induced by RU486 would prevent a PR polypeptide bound to RU486 from heterodimerization with another PR polypeptide bound to R5020. To evaluate this, PR-A and PR-B that were separately bound to R5020 or RU486 in whole cells were mixed in vitro. PR-A–RU486 was capable of dimerization with PR-B–R5020, and this was demonstrated for heterodimers both formed in solution and bound to specific DNA. The capability to form heterodimers in vitro raises the possibility that the antagonist action of RU486 in vivo could in part be imposed in a dominant negative fashion through heterodimerization between one receptor subunit bound to an agonist and another bound to RU486.

Human progesterone receptors (PR)[1] in breast cancer cells belong to the steroid receptor family of ligand-dependent transcriptional activators. Members of this family, which contain separable functional domains for hormone and DNA binding, regulate specific gene expression by binding to cis-acting hormone responsive elements (HREs) of target genes (Evans, 1988; Beato, 1989). Although the precise mechanisms by which hormonal ligands activate receptors remain unresolved, activation appears to be a multistep process. In the absence of hormone, steroid receptors are associated with heat shock protein 90 (hsp 90) and perhaps other hsps, depending on the class of receptor, to form a nontransformed, oligomeric complex that is unable to bind to DNA. Several studies suggest that an early step in response to hormone binding is dissociation of receptors from the oligomeric complex (Pratt, 1990). Upon dissociation of the nontransformed receptor complex, several different classes of steroid receptors have been shown to form dimers both in solution (DeMarzo et al., 1991; Fawell et al., 1990; Notides et al., 1981; Rodriguez et al., 1990; Wrange et al., 1989; Skafar, 1991a) and when bound to specific HREs (Beato et al., 1988; El-Ashry et al., 1989; Kumar & Chambon, 1988; Tsai et al., 1988). Moreover, sequence regions responsible for dimerization have been localized to the C-terminal steroid binding domain, and studies with receptor mutants have indicated that dimerization is an obligatory intermediate step that occurs prior to DNA binding (Fawell et al., 1990; Guchion-Mantel, 1989).

PR is somewhat unusual among the steroid hormone class of receptors since it is synthesized as two different sized polypeptides termed PR-A (94 kDa) and PR-B (120 kDa) that are expressed in approximately equal amounts in T47D breast cancer cells (Horwitz et al., 1985). PR-A is missing 164 N-terminal amino acids that are present in PR-B; thus, both receptor forms contain identical DNA-binding and C-terminal steroid binding domains (Kastner et al., 1990). Our laboratories and others have demonstrated that binding of human PR to a progesterone response element (PRE) is hormone-dependent and that three dimeric species bind to a PRE: dimers composed of AA, AB, and BB subunits (Edwards et al., 1989; El-Ashry et al., 1989; Meyer et al., 1990; Klein-Hitpass et al., 1991). We have recently shown by a coimmunoprecipitation assay that PR-A is capable of dimer-

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[1] Abbreviations: hsp, heat shock protein; HRE, hormone response element; PRE, progesterone response element; PR, progesterone receptor(s); PR-A, A form of progesterone receptor; PR-B, B form of progesterone receptor; MAb, monoclonal antibody.

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ization in solution with PR-B and that solution dimerization correlates with the dissociation of hsp 90 and the ability of PR to bind to specific DNA (DeMarzo et al., 1991). This suggests that dimerization in solution is required for PR binding to specific DNA and that hsp 90 may act indirectly to inhibit DNA binding by blocking dimerization.

The steroid analogue RU486 is a progesterone antagonist that is an important clinical compound as well as a useful tool for probing normal mechanisms of PR action (Philibert et al., 1984; Baulieu, 1989). Although receptor mechanisms responsible for the antagonist action of RU486 have been the subject of several investigations, the exact step or steps in the receptor activation pathway that are impaired by RU486 remain unresolved. Several reports have suggested that RU486, added to PR both in vivo and in vitro, does not impair the activation steps of transformation (dissociation from the non-DNA binding multiprotein complex) or binding to HREs. Accordingly, a step(s) subsequent to PR DNA binding, presumably interactions with other transcription factors or coregulators, has (have) been suggested to be the primary lesion responsible for the failure of RU486-bound PR to activate transcription (Bagchi et al., 1988; El-Ashry et al., 1989; Guiochon-Mantel et al., 1988; Klein-Hitpass et al., 1991; Meyer et al., 1990). Mechanistic studies revealing how PR transactivation function is inhibited by RU486 are lacking, however, and discrepancies remain since studies with calf uterine PR (Moudgil & Hurd, 1987) and rabbit uterine PR (Renoir et al., 1989, 1990) have suggested that RU486 does impair PR transformation. Because of the importance of dimerization as an intermediate regulatory step in the receptor activation pathway, we presently compared the effect of RU486 with that of the progestin agonist R5020 on solution dimerization of human PR. We also investigated whether it is possible for a PR polypeptide bound to RU486 to form a heterodimer in vitro with another PR polypeptide bound to R5020.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]R5020 (promegestone; [17-*methyl*-^{3}H]-17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; 87 Ci/mmol) and unlabeled R5020 were obtained from DuPont NEN products. [^{3}H]RU486 ([6,7-^{3}H]-17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-prop-1-ynylestra-4,9-dien-3-one; 37 Ci/mmol) and unlabeled RU486 were gifts from Roussel-UCLAF (Romainville, France). Klenow DNA polymerase and poly(dA-dT)·poly(dA-dT) were obtained from Pharmacia/LKB, and [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol) were from ICN. ^{35}S-Labeled protein A was from Amersham. Monoclonal antibodies (MAb) AB-52 and B-30 (mouse IgG_1) were raised against purified human PR (Estes et al., 1987). AB-52 recognizes both PR-A and PR-B while B-30 recognizes PR-B only.

Cell Culture, Baculovirus Expression of PR, and Receptor Purification. PR-rich T47D human breast cancer cells were cultured as described (Estes et al., 1987; El-Ashry et al., 1989). *Spodoptera frugiperda* (Sf9) insect cells were grown at 27 °C in Grace's insect medium (GIBCO) supplemented with 3.3 g/L yeastolate (GIBCO), 3.3 g/L lactalbumin hydrolysate (GIBCO), 25 μg/mL Gentamicin (Irvine), and 10% fetal bovine serum (Hyclone). For infection with recombinant baculovirus vectors, Sf9 cells either were plated as attachment cultures in 150-mm culture dishes (Falcon) at a density of 25 $\times$ 10^6 cells/dish or were maintained in suspension cultures in spinner flasks at a density of 1 $\times$ 10^6 cells/mL. Attachment cells were infected with recombinant viruses at an MOI of 1.0 for 1 h at 27 °C. The culture medium was removed and replaced with fresh medium, and cells were then incubated for another 48 h and harvested. Cells in suspension culture were infected with recombinant viruses at an MOI of 2 for 1 h and then incubated for another 48 h at 27 °C. Construction of recombinant baculoviruses expressing either intact full-length A or intact full-length B isoforms of human PR and characterization of their functional properties have been previously detailed (Christensen et al., 1991).

Buffers used for cellular extraction and immunoprecipitation of PR were derived from TEG (10 mM Tris–OH, pH 7.4, 1 mM EDTA, and 10% glycerol). T47D cells were homogenized in TEDG (TEG plus 1 mM dithiothreitol) on ice in the presence of a cocktail of protease inhibitors as described (Estes et al., 1987). To activate PR in the intact cell (in vivo), T47D cell cultures were incubated for 1 h at 37 °C with R5020 or RU486 (20 nM). PR were then extracted from nuclei with 0.5 M NaCl as described (El-Ashry et al., 1989). Recombinant receptors, expressed from baculovirus, were activated by incubation of Sf9 insect cells for 2 h at 27 °C with 100 nM hormone. Whole-cell extracts of infected Sf9 cells were prepared by performing cell lysis in TEDG containing protease inhibitors and 0.5 M NaCl. Samples were then clarified by centrifugation at 100000*g* for 30 min. Activated receptors (either in vivo or in vitro), from either cell-type, were dialyzed at 4 °C against TEDG buffer to reduce the salt concentration prior to immunoprecipitation and DNA binding assays. For both T47D and Sf9 cell extracts, the number of steroid receptor binding sites was determined by dextran-coated charcoal assay as previously described (Estes et al., 1987), and protein concentrations were measured by the method of Bradford (1976).

For mixing experiments in which separately expressed PR-A and PR-B from baculovirus were examined for their ability to form PR-A·PR-B heterodimers in vitro, it was first necessary to eliminate minor receptor degradation products that are produced with baculovirus-expressed PR-B (Christensen et al., 1991). This was accomplished by MAb immunoaffinity purification using the B-30 MAb attached covalently to Affigel-10 (Bio-Rad). PR-B immobilized to Affigel-10 was washed with 0.5 M NaCl to dissociate most of the receptor degradation products, and then the intact full-length PR-B was eluted by exposure to alkaline pH (10 mM NaOH) followed by immediate neutralization with 100 mM Tris-HCl (pH 7.4) containing the following additional ingredients: 10 mM $MgCl_2$, 10 mM DTT, 12.5% glycerol, 1 mM EDTA, 0.2 mM EGTA, 25 mM NaCl, and 50 μM $ZnCl_2$. This elution method maintains PR in a biologically active form (Estes et al., 1987). Immunoaffinity-purified PR-B is >70% pure as judged by silver-stained SDS gels.

Coimmunoprecipitation of PR-A with a PR-B-Specific MAb. MAb-coated protein A–Sepharose was prepared as previously described (DeMarzo et al., 1991). Briefly, resins were prebound with a bridging antibody, rabbit anti-mouse IgG (Cappel), followed by binding of receptor-specific MAbs (AB-52 or B-30) to the immobilized rabbit anti-mouse IgG. For immunoprecipitation of PR from T47D cells, aliquots of cell extracts (amounts of PR are indicated in the figure legends) were incubated with MAb-coated protein A–Sepharose beads on an end-over-end rotator for 4 h at 4 °C. Immobilized receptor complexes were then washed 3 times by centrifugation in low ionic strength TEG (or TEG plus NaCl as stated in the figure legends) followed by transfer to a new microcentrifuge tube and one additional wash. To determine the level of nonspecific binding, parallel immu-

noprecipitations were performed with protein A–Sepharose that had been precoated with rabbit anti-mouse IgG only. In experiments to examine solution dimerization of PR-A–RU486 with PR-B–R5020, the two receptor isoforms were expressed separately from baculovirus vectors. PR-B was purified as described above and mixed in vitro with PR-A that was prepared as a whole-cell extract. After mixing and incubation for 10 min at 0 °C, receptors were then immunoprecipitated as above, except that incubation with MAb-coated protein A–Sepharose was shortened to 1 h at 4 °C.

SDS Gel Electrophoresis and Western Immunoblotting. Immobilized PR complexes were eluted from protein A–Sepharose with a 2% SDS loading buffer, and SDS–polyacrylamide gel electrophoresis and Western immunoblotting were carried out as previously described (ElAshry-Stowers et al., 1988) using ^{35}S-labeled protein A and autoradiography of dried nitrocellulose as the detection method. Dried nitrocellulose blots were scanned directly for ^{35}S in protein bands with the BioScan 200 image scanner (BioScan, Washington, DC), or with the Series 400 Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Gel Mobility Shift. Gel mobility shift assays were performed as previously described (DeMarzo et al., 1991; El-Ashry et al., 1989) with minor modifications. PR (femtomoles of receptors are indicated in the figure legends) in crude cell extracts or collected directly from sucrose gradient fractions were incubated as indicated in the figure legends with a double-stranded synthetic oligonucleotide corresponding to the distalmost progesterone/glucocorticoid response element (−189 to −162 from the transcription start site) of the mouse mammary tumor virus (referred to as a PRE). The PRE was end-labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol, ICN) by Klenow DNA polymerase to a specific activity of approximately 20 000–30 000 cpm/0.1 ng of DNA. The DNA binding reaction contained 0.1–0.3 ng of labeled PRE and 2 μg of poly(dA-dT)·poly(dA-dT) as nonspecific competitor DNA in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM dithiothreitol, 2 mM $MgCl_2$, 0.5 μg/μL gelatin, and 10% glycerol. The DNA binding reactions (20 μL total) were subjected to electrophoresis and autoradiography as described (El-Ashry et al., 1989). Quantitation of receptor-bound ^{32}P-labeled PRE was carried out by direct scanning of dried gels using the Series 400 Molecular Dynamics PhosphorImager.

Sucrose Density Gradient Analysis of PR. Linear 5–20% sucrose gradients (5 mL) were prepared in TEDG containing 0.3 M NaCl. PR were activated with unlabeled R5020 or RU486 in whole cells (T47D or Sf9) and extracted with 0.5 M NaCl as described above. Extracts were diluted with TEDG to 0.3 M NaCl. Unlabeled hormones bound to PR were partially exchanged in vitro with radiolabeled hormones by incubation with either [^3H]R5020 or [^3H]RU486 (20 nM) for 16 h at 4 °C. For experiments using the B-30 MAb to shift receptors in the gradients, each sample was divided into aliquots. One aliquot was left untreated; the others were incubated with MAb (100-fold excess B-30/PR by weight) for 4 h at 4 °C. Each sample was brought to 250 μL by addition of TEDG (plus protease inhibitors) and then layered on preformed gradients. Gradients were centrifuged at 250000g for 16 h; 200-μL fractions were collected from a puncture hole in the bottom of the gradient tubes, and 25-μL aliquots were counted for radioactivity in 5.0 mL of 3A70B liquid scintillation fluid (Research Products International, Mount Prospect, IL). A small aliquot (2–5 μL) of receptor-containing fractions was then submitted directly to the gel mobility shift assay, or the entire remaining sample was submitted to immune isolation and Western immunoblotting as described in the figure legends.

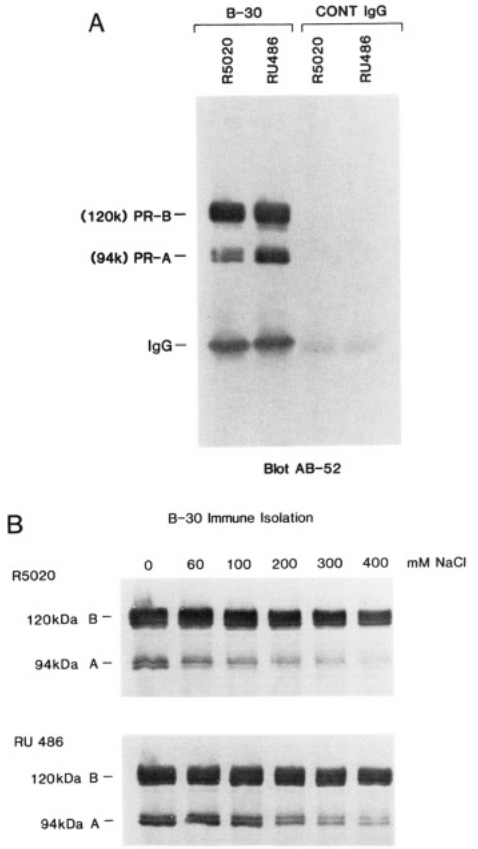

FIGURE 1: RU486 binding to PR in vivo enhances solution dimerization of PR as detected by coimmunoprecipitation assay. (A) Equal amounts of PR (6–8 pmol based on a hormone binding assay), that were activated in vivo with R5020 or RU486 and extracted from nuclei, were immunoprecipitated with either the PR-B-specific MAb B-30 or a control unrelated antibody as described under Experimental Procedures using protein A–Sepharose as an immunoabsorbant. Protein A–Sepharose was washed in TEG buffer, and immobilized receptors were extracted and detected by Western immunoblot with another MAb, AB-52, which recognizes both PR-A and PR-B. IgG is the heavy chain of the antibodies that coelute from protein A–Sepharose. Quantification of the ratio of PR-A to PR-B was carried out by direct scanning of immunoblots (BioScan 200 image scanner) from $N = 6$ experiments and a total of eight separate determinations. (B) Identical procedure as in (A) except that immobilized PR were washed with TEG containing the indicated concentrations of NaCl.

RESULTS

RU486 Addition to Whole Cells Promotes More Efficient Dimerization of PR than the Progestin Agonist R5020. To examine the effect of RU486 on PR dimerization in solution, we employed a previously developed coimmunoprecipitation assay (DeMarzo et al., 1991) that detects dimerization of PR-A with PR-B. The assay involves immunoprecipitation of PR from cell extracts with the PR-B-specific MAb B-30, using protein A–Sepharose as an immunoabsorbent. Immobilized PR is then eluted from protein A–Sepharose and analyzed by Western immunoblot with a different MAb, AB-52, that recognizes both the A and B isoforms of PR (Estes et al., 1987). Coimmunoprecipitation of PR-A indicates the presence of PR-A·PR-B heterodimers in solution that are stable to the conditions of immunoprecipitation. Figure 1A shows the results of this assay with receptors that were bound to

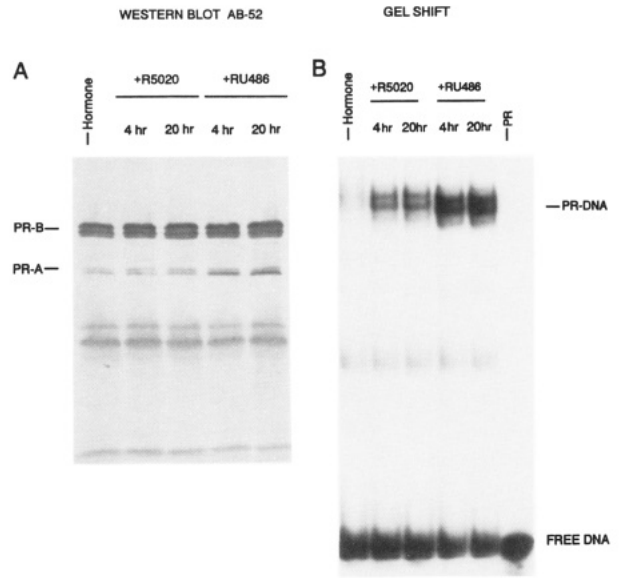

FIGURE 2: RU486 binding to PR in vitro enhances both solution dimerization of PR and binding activity for a PRE. (A) PR, prepared as a 0.5 M NaCl extract of nuclear fractions from non-hormone-treated T47D cells, were dialyzed for 2 h against TEDG and then incubated with or without R5020 (100 nM) or RU486 (100 nM) for the times indicated. Receptors were then immunoprecipitated with the PR-B-specific MAb B-30 and subjected to Western immunoblot with AB-52 as in Figure 1A. The relative amount of PR-A coisolated with PR-B was quantified by direct scanning of the Western blot for the ratios of PR-A to PR-B (Molecular Dynamics PhosphorImager). Scanning of dried nitrocellulose filters from seven determinations from three separate experiments indicated that the average amount of PR-A coisolated per unit of PR-B was 12% for non-hormone-treated receptors, 13% for R5020-bound PR, and 23% for RU486-bound PR. (B) PR samples were prepared as in (A) up to the step of immunoprecipitation. NaCl extracts of T47D cell nuclei were dialyzed against TEDG, incubated with or without R5020 or RU486, and then analyzed for binding to a ^{32}P-end-labeled PRE oligonucleotides by the gel mobility shift assay. The PR–DNA binding reaction was for 1 h at 4 °C. Scanning of dried gel mobility shift gels (Molecular Dynamics PhosphorImager) from seven determinations from two separate experiments revealed an average 5-fold greater PR–DNA binding in the presence of R5020 over no hormone, and an additional 2.4-fold increase in the presence of RU486.

ligands in vivo by addition of either R5020 or RU486 to intact T47D cells and then extracted from nuclei. Significant amounts of PR-A are coimmunoprecipitated with PR-B after treatment with either ligand; however, the extent of PR-A that complexes with PR-B is substantially greater in the presence RU486. Figure 1A (right panel) also shows that PR-A associates specifically with PR-B, since A receptors are not immunoprecipitated by an unrelated control antibody. We have quantitated the effect of RU486 on solution dimerization of PR by directly scanning Western blots to determine the relative amounts of PR-A that are coimmunoprecipitated with PR-B. From replicate experiments similar to that in Figure 1A, RU486 enhances solution dimerization of PR-A with PR-B by an average of approximately 2-fold over that obtained with R5020. From six separate experiments, the average relative amount of PR-A coimmunoprecipitated per unit of PR-B was 27% (SEM = 3.2) in the presence of R5020 and 49% (SEM = 4.2) in the presence of RU486. It should be pointed out that PR-A and PR-B are capable of forming AA and BB homodimers as well as AB heterodimers when complexed to DNA (El-Ashry et al., 1989, Meyer et al., 1990; Klein-Hitpass et al., 1991). However, the relative distribution of the three dimers in solution in vivo is not known. If the A and B proteins were to interact randomly in solution, binomial distribution predicts a molar ratio for receptor dimers of 1 (AA):2 (AB):1 (BB) since A and B are expressed in approximately equal amounts in T47D cells (El-Ashry et al., 1988). In support of this distribution ratio, the intermediate-mobility AB heterodimers are the predominant form of PR complexed to DNA (El-Ashry et al., 1989; Figures 2, 3, and 7). With the assumption that solution dimers in vivo are in the ratio of 1 (AA):2 (AB):1 (BB), the PR-B-specific MAb (B-30) will immunoprecipitate two AB dimers for every one BB dimer. Therefore, for every 2 units of PR-B, a maximum of 1 unit of PR-A can be coimmunoprecipitated by B-30 if all PR are dimeric. If dimers are present with monomers, then less PR-A will be coimmunoprecipitated per unit of PR-B. The maximal amount of coisolated PR-A, therefore, would be 50% of the isolated PR-B. Thus, our quantitation of the relative amounts of PR-A that coimmunoprecipitate with PR-B suggests that virtually all PR bound to RU486 are dimeric (i.e., PR-A = 49% of immunoprecipitated PR-B), yet under identical conditions only half of PR are dimeric when bound to R5020 (i.e., PR-A = 27% of immunoprecipitated PR-B).

To examine whether the more efficient solution dimerization could be due to RU486 promoting an increased stability of PR dimers, the coimmunoprecipitation assay was performed in the presence of increasing concentrations of NaCl. As shown in Figure 1B, solution dimerization of PR is sensitive to NaCl concentration (DeMarzo et al., 1991), since the relative amount of PR-A that coimmunoprecipitates with PR-B decreases with increasing NaCl concentrations. Figure 1B also shows some differences in salt sensitivity between PR dimers bound with R5020 (upper panel) and RU486 (lower panel). PR-A·PR-B dimers bound to RU486 are relatively resistant to NaCl concentrations up to a concentration of 100 mM, yet a significant amount of dissociation of PR-A·PR-B dimers occurs at a NaCl concentration as low as 60 mM when PR is bound to R5020. At higher concentrations of NaCl (200–400 mM), considerable dissociation of PR-A·PR-B dimers occurs with either ligand; however, substantial amounts of dimers are retained at 400 mM NaCl only when receptors are bound to RU486.

It should be noted that receptors activated by hormone addition to the intact cell in Figure 1 were extracted from nuclei with 0.5 M NaCl and dialyzed prior to coimmunoprecipitation assay. Because PR-A·PR-B dimers are not stable in 0.5 M NaCl (Figure 1B), this suggests that dimers dissociate upon nuclear extraction and reassociate during dialysis. Figure 1B also shows that PR-A migrates as a doublet on SDS gels. The slower mobility (upper) band represents PR-A that has undergone hyperphosphorylation in response to hormone (Sheridan et al., 1988; Beck et al., 1992). It is of interest that with receptors bound to R5020, the hyperphosphorylated PR-A band that dimerizes with PR-B is more resistant to NaCl dissociation than the non-upshifted PR-A form, suggesting that phorphorylation may contribute to dimer stability (DeMarzo et al., 1991). By contrast, both PR-A bands show the same salt sensitivity when PR is bound to RU486, and in general, the underphosphorylated PR-A band is responsible for most of the increased dimerization of PR-A with PR-B that occurs when receptors are bound to RU486 (Figures 1 and 2A). On the basis of these solution dimerization assays, we conclude that RU486 does not impair dimerization of PR; rather, it enhances dimerization compared with the progestin agonist R5020; this is reflected by an approximately 2-fold increase in the amount of measurable PR-A·PR-B dimers and by a somewhat increased stability of dimers against NaCl dissociation.

RU486 Addition in Vitro Promotes More Efficient Solution Dimerization and DNA Binding Activity of Human PR than R5020. Experiments in Figure 1 were conducted with receptors that were bound with ligands in vivo (intact T47D cells), and thus the possibility exists that the effects of RU486 could be due to secondary cellular processing events and not directly to the binding of RU486 to the receptors. To examine more directly the influence of RU486 binding on solution dimerization, nuclear PR from non-hormone-treated cells were stripped free of hsp 90 and subsequently treated in vitro with either R5020 or RU486 (Figure 2). A substantial fraction of PR in T47D cells are tightly bound to nuclei in the absence of hormone, and these receptors require high salt for extraction and sediment as 4 S species on density gradients (Bagchi et al., 1990). Figure 2A shows that PR stripped free of hsp 90 in this manner are capable of forming some solution PR-A·PR-B dimers in the absence of added ligand. This is in contrast to nonactivated cytosol receptors that remain complexed with hsp 90 in low ionic strength buffers that we have shown previously do not form solution dimers (DeMarzo et al., 1991). Salt-induced dissociation of hsp 90 therefore appears to be sufficient to allow at least some receptor dimerization to occur in the absence of hormone (Figure 2A). Addition of R5020 in vitro for 4 or 20 h at 4 °C does not produce a further detectable increase in the extent of PR-A dimerization with PR-B (Figure 2A). By contrast, RU486 addition in vitro does result in an approximate 2-fold increase in the amount of PR-A that coimmunoprecipitates with PR-B (Figure 2A). In Figure 2B, these same receptor preparations were analyzed by gel mobility shift assay for their ability to bind to a PRE. In the absence of hormone, PR stripped free of hsp 90 displays little, if any, DNA binding activity (Figure 2B). R5020 addition in vitro results in a substantial induction of DNA binding activity (5-fold) over the barely detectable PR–DNA complexes obtained without hormone. Interestingly, R5020 induction of PR–DNA binding occurs in the absence of a detectable effect on PR dimerization in solution (compare R5020 in Figure 2A and Figure 2B). By contrast, RU486 addition increases DNA binding another 2.4-fold over that obtained with R5020 (Figure 2B). Thus, the 2-fold-enhanced PR dimerization promoted by RU486 correlates well with the ≈2–3-fold higher DNA binding activity observed here and in earlier studies (Bagchi et al., 1988, 1990; El-Ashry et al., 1989). Figure 2B also shows that PR forms three closely spaced complexes with the PRE DNA that have been shown in earlier studies to contain receptor dimers composed of AA, AB, and BB subunits (El-Ashry et al., 1989; Klein-Hitpass et al., 1991). All three complexes in Figure 2B exhibit slightly faster mobilities when PR is bound in vitro to RU486 compared with PR bound to R5020. In an earlier study, we reported that PR–DNA complexes have a faster mobility when receptors used in the assay are bound in the intact cell with RU486 compared with R5020 (El-Ashry et al., 1989). The results in Figure 2B show that RU486 binding to PR in vitro also produces the alteration in receptor structure that results in a faster electrophoretic mobility on gel-shift assays.

Thus, the fact that RU486 binding to PR both in vitro and in vivo promotes more efficient solution dimerization of PR than R5020 suggests that RU486 interaction directly alters the dimerization properties of PR. An unexpected observation is that R5020 addition in vitro does not significantly increase the extent of PR-A·PR-B solution dimerization over that obtained by salt dissociation of the PR–hsp 90 complex, yet R5020 addition is required for induction of DNA binding. This suggests that for human PR, dimerization may be dependent on dissociation from hsp 90 and that continuous ligand binding is necessary to induce a further conformational change in the receptor dimer required for DNA binding.

The Increased Sedimentation Coefficient of PR Bound to RU486 Is Not the Result of Stabilization of Receptor Dimers. Earlier studies demonstrated that salt-treated human PR, bound with RU486, exhibits a faster sedimentation rate (6 S) on sucrose density gradients than PR bound with R5020 which sediment at 4 S (El-Ashry et al., 1989, Mullick & Katzenellenbogen, 1986). Moreover, the 4 S human progesterone receptor bound to R5020 on sucrose density gradients has been shown previously to be a receptor monomer (Wei et al., 1987). Since RU486 promotes more efficient solution dimerization of PR (Figures 1 and 2) than R5020, we questioned whether the 6 S sedimentation of the PR–RU486 complex might represent a PR dimer that is stable to gradient centrifugation. To test this, we performed a variation of the coimmunoprecipitation assay. T47D cell extracts containing activated PR were bound prior to gradient centrifugation with the PR-B-specific MAb B-30. Binding of B-30 will increase the sedimentation rate of PR-B, and if PR-A·PR-B heterodimers are stable, PR-A should co-shift on the gradients with the B-30·PR-B complex. Figure 3 shows the characteristic 6 S (peak 1) and 4 S (peak 2) sedimentation of PR–RU486 and PR–R5020 complexes, respectively, in the absence of added MAb. Addition of the B-30 MAb shifts approximately half of the receptors to a faster sedimenting form when they are bound with either ligand, RU486 (peak 3) or R5020 (peak 4). Interestingly, the faster sedimentation of the PR–RU486 complex is maintained even when receptors are bound to the B-30 MAb (compare peaks 3 and 4 in Figure 3A). To determine whether any PR-A remains complexed to PR-B during the gradient centrifugation, peak receptor fractions shifted by B-30 (PR–RU486, peak 3; and PR–R5020, peak 4) were immunoprecipitated and then subjected to a Western immunoblot with AB-52. As shown in Figure 3B, no detectable PR-A is co-shifted with the B-30 MAb whether receptors are bound to RU486 (peak 3) or R5020 (peak 4). All the A receptor remains in the non-MAb-shifted peaks 5 and 6. Thus, a complete separation of B and A receptors occurs on sucrose density gradients, even in the presence of RU486. As expected, receptor peak fractions designated 1 (PR–RU486, peak 1) and 2 (PR–R5020, peak 2), to which no MAb is added, contain approximately equal amounts of PR-A and PR-B (Figure 3B). These results demonstrate that transformed receptors bound to RU486 do not form dimers that are stable to gradient centrifugation, despite the ability of RU486 to increase the efficiency of PR dimerization in solution (Figures 1 and 2). This indicates that the 6 S sedimentation reflects some other structural alteration in PR monomers.

Figure 3C shows that the monomeric PR, collected from gradient fractions, can readily re-form dimers upon dilution and will bind to a PRE. Collected gradient fractions, after dilution in DNA binding buffer, produce three specific receptor–DNA complexes when incubated with the ^{32}P-labeled PRE (Figure 3C). The three complexes correspond as previously shown to receptor dimers composed of BB (complex 1), AB (complex 2), and AA (complex 3) subunits (El-Ashry et al., 1989). It should be noted that the DNA binding activities in the collected gradient fractions in Figure 3C are coincident with the ligand binding activity for both the 4 S (PR–R5020) and 6 S (PR–RU486) receptors (compare Figure 3A and Figure 3C). Coimmunoprecipitation of the gradient fractions indeed confirmed that PR-A·PR-B dimers do re-

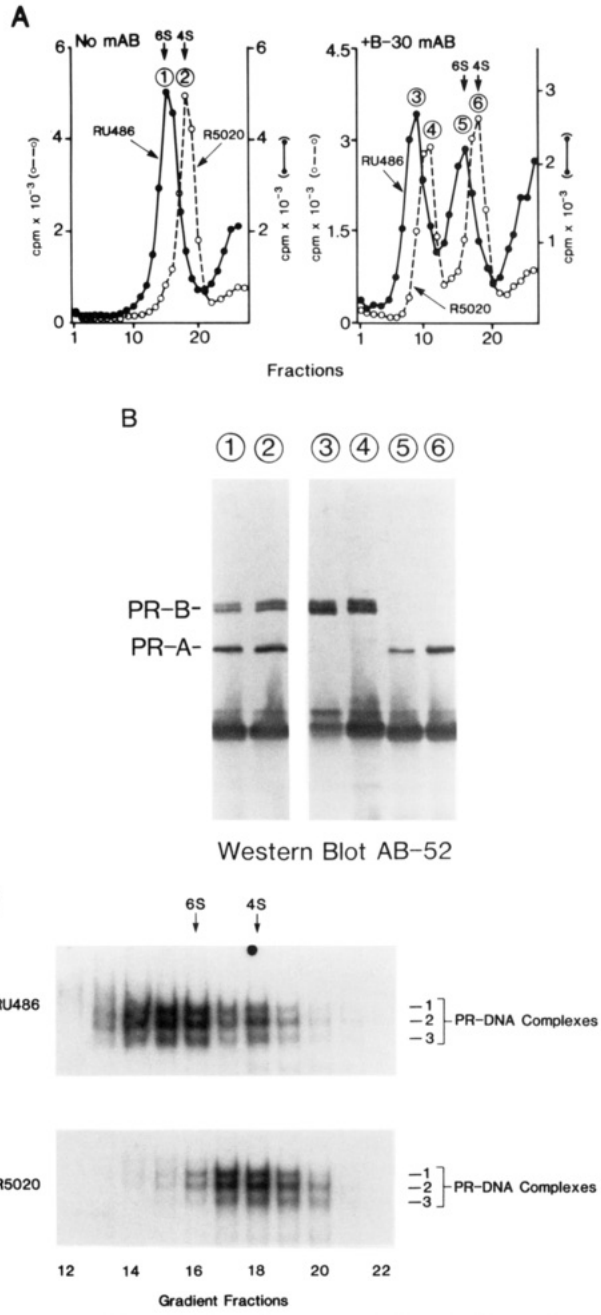

FIGURE 3: RU486 binding does not stabilize PR dimers during gradient centrifugation. (A) High-salt (0.5 M NaCl) nuclear extracts containing PR, that were activated with R5020 or RU486 in T47D cells, were diluted to 0.3 M NaCl in TEDG buffer and incubated for 16 h at 0 °C with either 20 nM [^{3}H]R5020 or 20 nM [^{3}H]RU486 to partially exchange unlabeled ligands with ^{3}H-labeled ligands. An aliquot of receptor–^{3}H-ligand complexes was then incubated with the PR-B-specific MAb B-30 for 4 h at 4 °C. Both nontreated and B-30-treated PR were then centrifuged on sucrose density gradients containing 0.3 M NaCl. Fractions were collected, and aliquots were counted for ^{3}H-ligand bound to receptor. Peak receptor fractions were assigned a number (1–6). (B) The remaining aliquots from collected receptor fractions in (A) were immunoprecipitated with either receptor-specific MAb AB-52 using protein A–Sepharose as the immunoabsorbent (peaks 1, 2, 5, and 6) or protein A–Sepharose prebound with rabbit anti-mouse IgG secondary antibody (peaks 3 and 4 already have B-30 MAb bound to PR). Immobilized receptors were then eluted from protein A–Sepharose and subjected to Western immunoblot with AB-52. (C) The 6 S PR–[^{3}H]RU486 complexes and 4 S PR–[^{3}H]R5020 complexes were separated by sucrose density gradient centrifugation as in (A) without addition of B-30 MAb. Aliquots (5 μL) of the collected fractions were diluted 4-fold in DNA binding buffer and assayed by the gel mobility shift assay for binding to the ^{32}P-labeled PRE. Specific DNA binding was carried out for 30 min at room temperature.

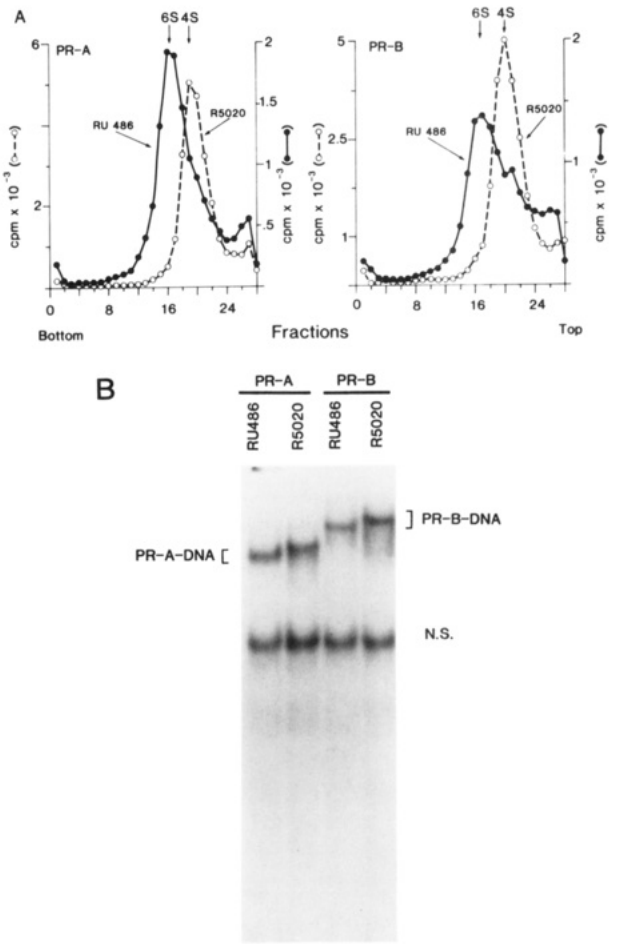

FIGURE 4: RU486 binding to baculovirus-expressed PR-A and PR-B promotes increased sedimentation on sucrose gradients and increased electrophoretic mobility of receptor–DNA complexes. (A) Baculovirus-expressed PR-A and PR-B were each bound separately with unlabeled R5020 or RU486 in Sf9 cells and then prepared as 0.5 M NaCl extracts of whole-cell lysates. Extracts were diluted to 0.3 M NaCl and incubated for 16 h at 4 °C with the homologous ^{3}H-ligand (100 nM) to partially exchange with unlabeled ligand. Receptors were then analyzed by centrifugation on sucrose gradients as in Figure 3. (B) The same extracts as in (A) were dialyzed and subjected to the gel mobility shift assay. The DNA binding reaction was for 1 h at 0 °C.

form upon dilution in DNA binding buffer (not shown). We conclude, therefore, that dissociation of PR dimers during gradient centrifugation must be due to the combined effects of ultracentrifugation and exposure to NaCl, since salt treatment alone in solution is insufficient to completely disrupt PR dimers when the receptor is bound to RU486 (Figure 1B).

RU486 Induces a Structural Change in Baculovirus-Expressed PR Similar to That Observed with Endogenous PR. If a conformational change in PR is induced directly by RU486 binding, then recombinant human PR expressed in a heterologous system should be affected similarly by RU486. PR-A and PR-B were separately expressed from baculovirus vectors in Sf9 insect cells as full-length nonfusion proteins. As previously described, both PR isoforms are functional in terms of hormone binding and hormone-dependent DNA binding activities (Christensen et al., 1991). Figure 4A shows the sedimentation of baculovirus-synthesized PR-A and PR-B on salt-containing sucrose gradients when each is bound to [^{3}H]R5020 or [^{3}H]RU486. Each receptor isoform bound to RU486 sediments at approximately 6 S while the same two isoforms bound to R5020 sediment at 4 S (Figure 4A). Recombinant PR-A and PR-B expressed from baculovirus and bound to R5020 or RU486 were also compared for their

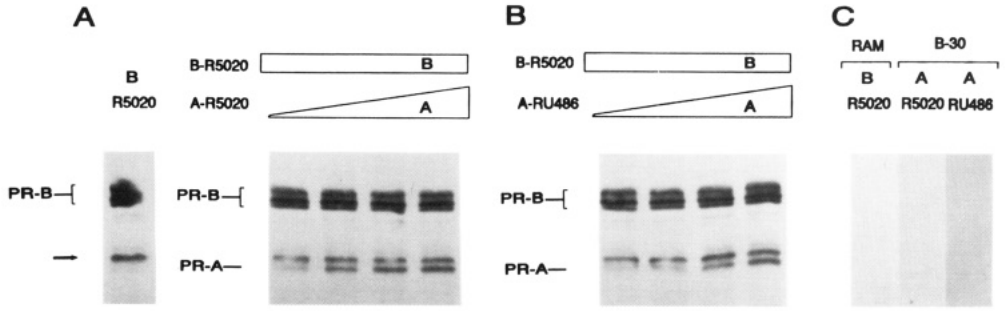

FIGURE 5: PR bound to RU486 is capable of solution heterodimerization with PR bound to R5020. (A) Baculovirus-expressed PR-B that was bound to R5020 in Sf9 cells was immunoaffinity-purified with the PR-B-specific MAb B-30 as described under Experimental Procedures. PR-B·R5020 was then mixed with an Sf9 whole-cell extract from noninfected cells (extract used as nonspecific carrier protein) and reisolated with MAb B-30; the eluted receptor was subjected to a Western immunoblot with MAb AB-52. The arrow to the left of the first lane of panel A indicates the position of a proteolytic receptor fragment that contaminates the PR-B preparation and is slightly larger in molecular mass than authentic PR-A. This is a minor fraction of total PR-B and is present in constant amounts in all the lanes in which authentic PR-A was added. A constant amount of the immunoaffinity-purified PR-B bound with R5020 (600 fmol) was mixed in vitro for 10 min at 0 °C with increasing amounts (150, 300, 600, and 1200 fmol) of baculovirus-expressed authentic PR-A that was bound in vivo to either R5020 or (B) RU486. Samples were then immunoprecipitated with MAb, B-30, as in Figures 1 and 2, except that the incubation time with MAb-coated protein A–Sepharose was shortened to 1 h at 4 °C. Immobilized receptors were washed in TEG as in Figure 1A, eluted, and subjected to Western immunoblot with AB-52. (C) Immunoprecipitations with control antibodies: PR-B–R5020 (600 fmol) immunoprecipitated with rabbit anti-mouse IgG (RAM) and PR-A–R5020 (1200 fmol) and PR-A–RU486 (1200 fmol) immunoprecipitated with MAb B-30. Protein A–Sepharose beads were washed, eluted, and Western-blotted with AB-52 as in (A) and (B).

specific DNA binding properties by a gel mobility shift assay. As shown in Figure 4B, separately expressed PR-A and PR-B each form a single complex with a PRE, and the larger molecular weight B receptor as expected exhibits a slower mobility than the A receptor. When each receptor isoform is bound to R5020 or RU486 and compared side-by-side, PR-A bound to RU486 migrates slightly faster than PR-A bound to R5020, and the same relationship is observed with PR-B (Figure 4B). The gel-shift experiment in Figure 4B was performed with PR in whole-cell extracts of infected Sf9 insect cells. A similar experiment was performed with immunoaffinity-purified baculovirus-produced PR-B, and RU486 binding continued to promote the increased mobility of the PR–DNA complex compared with receptors bound to R5020 (data not shown). Thus, the fact that RU486 promotes the same alterations with baculovirus-produced PR (both in crude cell extracts and with purified PR) as with endogenous mammalian PR suggests that RU486 binding itself, and not secondary or cell-specific factors, is responsible for the observed alterations in both hydrodynamic and electrophoretic properties of PR.

Receptors Bound to RU486 Are Capable of Heterodimerization in Solution with Receptors Bound to R5020. Since our results suggest that RU486 induces a distinct conformation in human PR, we next questioned whether this altered conformation might prevent receptors bound to RU486 from recognizing and dimerizing with receptors bound to R5020. To examine this, the A and B receptor isoforms were each expressed separately from baculovirus vectors, and were then bound in Sf9 insect cells with either R5020 or RU486. Extracted receptors were mixed in vitro and tested for their ability to form PR-A·PR-B heterodimers in solution by the coimmunoprecipitation assay. Because baculovirus-produced PR-B typically yields some receptor degradation products (Christensen et al., 1991), PR-B for these mixing experiments was purified by MAb affinity chromatography with the PR-B-specific MAb B-30 in order to remove PR fragments. Purification removes all receptor degradation products except one that is slightly larger in apparent molecular weight on SDS gels than authentic PR-A (Figure 5A, lane 1). This PR-B fragment (indicated by the arrow in Figure 5A), however, is a minor fraction of the total intact PR-B. Increasing amounts of PR-A (prepared as a whole-cell extract) bound to either R5020 or RU486 were briefly mixed (10 min at 4 °C) with a constant amount of purified PR-B–R5020, and samples were submitted to the coimmunoprecipitation assay with the PR-B-specific MAb B-30. As shown in Figure 5A, substantial amounts of authentic PR-A–R5020 (indicated by the dash in Figure 5B) are coimmunoprecipitated with purified PR-B–R5020 in a dose-responsive fashion. Similar results are obtained when PR-A bound to RU486 is mixed in increasing amounts with a constant amount of purified PR-B bound to R5020 (Figure 5B). We have also mixed in the opposite direction; PR-B–RU486 with PR-A–R5020 or PR-A–RU486. These experiments produced amounts of solution heterodimers similar to that observed in Figure 5B (not shown). Figure 5C shows controls for the coimmunoprecipitation assay. PR-A is specifically complexed with PR-B, since no PR-A (bound to either R5020 or RU486) is immunoprecipitated by the B-30 MAb in the absence of added PR-B, or by an unrelated control antibody (RAM).

A potential problem with interpretation of this experiment is the possibility of ligand exchange between the mixed PR isoforms during the coimmunoprecipitation assay. Several observations and additional control experiments indicate that ligand exchange is minimal and does not likely account for the observed solution heterodimerization. First, the experimental conditions of the assay do not favor ligand exchange. Both R5020 and RU486 bind PR with high affinity and display slow rates of exchange at 0–4 °C (Horwitz, 1985). The condition for mixing in Figure 5 was 10 min at 0 °C followed by immunoprecipitation for 1 h at 4 °C. Second, to minimize exchange, excess free ligand was removed in some experiments by treatment with dextran-coated charcoal immediately prior to mixing of receptors. We found no difference in the relative amounts of either PR-A–RU486 or PR-A–R5020 complexed with PR-B–R5020, whether or not excess free ligand was removed (not shown). As an additional control for ligand exchange, baculovirus-expressed PR-B, bound to unlabeled RU486, was prebound with the B-30 MAb and then mixed in vitro with PR-A bound to [^{3}H]R5020, for a total time that was 2.5 times longer than that in the mixing experiment shown in Figure 5. The A and B receptors were then separated as in Figure 3 by density gradient centrifugation. By assaying for [^{3}H]R5020 bound to the shifted PR-B MAb complex, we estimated that, at most, 4% of the PR-B–RU486 complex becomes bound with [^{3}H]R5020. This level of ligand exchange occurs after a much longer time than the mixing of PR-A and PR-B in Figure 5 and is far too little to account for the observed

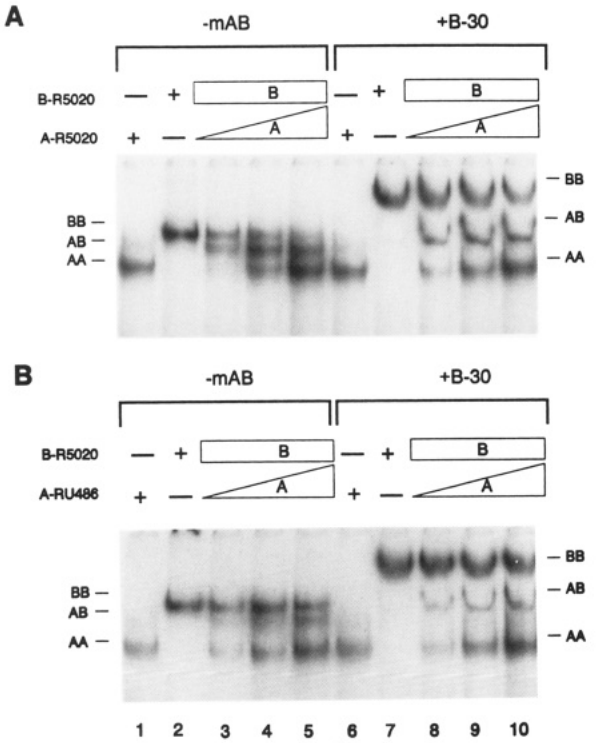

FIGURE 6: Detection of RU486·R5020 PR heterodimers bound to DNA. (A) Recombinant PR-A and PR-B, separately expressed from baculovirus, were activated in Sf9 cells with R5020. PR-A was prepared as a whole-cell extract, and PR-B was immunoaffinity-purified as described under Experimental Procedures. Each was then analyzed separately (20 fmol) for binding to ^{32}P-labeled PRE in a gel-shift assay (lanes 1 and 2, respectively). Since immunoaffinity-purified PR-B does not bind well to DNA in the absence of other cellular factors (S. A. Oñate, S. K. Nordeen and D. P. Edwards, unpublished results), PR-B–R5020 in lane 2 was mixed with Sf9 whole-cell extracts from noninfected Sf9 cells in amounts equivalent to that present in crude preparations of PR-A found in lane 1. A constant amount of PR-B–R5020 (20 fmol) was mixed with increasing amounts of PR-A–R5020 (lanes 3–5; 10, 20, and 40 fmol, respectively) for 10 min at 0 °C and then incubated with ^{32}P-labeled PRE. For all lanes, DNA binding was carried out for 15 min at 0 °C prior to electrophoresis. Lanes 6–10 represent the same reactions, respectively, as in lanes 1–5 except that the B-30 MAb was added for the last 5 min of the DNA binding reaction. (B) Identical experiment as in part A except PR-A was bound to RU486.

extent of PR-A dimerization with PR-B. We conclude, therefore, that PR bound to RU486 is capable of heterodimerization in solution with receptors bound to a progestin agonist.

PR–R5020·PR–RU486 Heterodimers Bind to Specific DNA. We next examined whether the PR-A–RU486·PR-B–R5020 heterodimers formed in solution are also capable of binding to a PRE. Whole-cell extracts containing PR-A expressed in Sf9 insect cells and bound in vivo to R5020 (Figure 6A, lanes 3–5) or to RU486 (Figure 6B, lanes 3–5) were mixed in vitro in increasing amounts with a constant amount of purified PR-B bound to R5020 and the samples submitted to the gel-shift assay. As shown in Figure 6, mixing of PR-A–R5020 (panel A) or PR-A–RU486 (panel B) with PR-B–R5020 results in the appearance of a third intermediate-mobility DNA complex. Moreover, more of the intermediate complex is detected as increasing amounts of PR-A are added. The appearance of a third intermediate complex is consistent with the formation of PR-A·PR-B heterodimers bound to DNA, whether PR-A is bound with RU486 or R5020. Although the intermediate complex is less predominant when PR-A–RU486 (panel B), compared to PR-A–R5020 (panel A), is the mixing partner, it is apparent that a PR isoform bound to RU486 can dimerize during DNA binding with another isoform bound to R5020. It also appears that the third intermediate DNA complex is composed primarily of authentic PR-A dimerized to PR-B and that the PR-B degradation fragment (Figure 5) does not contribute to this complex to any substantial amount. Evidence for this is provided by the fact that PR-B alone (Figure 6A,B, lane 2), which contains the fragment, generates only a single receptor–DNA complex. This indicates that the PR-B fragment either does not bind the PRE or is present in amounts that are too low for detection. A second faster mobility complex would be expected in lane 2 of Figure 6 if the fragment could bind to the PRE. Gel mobility shift assays in Figure 6A,B were also performed with the addition of the PR-B-specific MAb B-30. This results in a supershift of PR-B homodimers and PR-A·PR-B heterodimers, but not PR-A homodimers. Results with B-30 addition also demonstrate that purified PR-B contains no detectable authentic PR-A or PR-B degradation fragments bound to DNA (lane 7, PR-B with no added PR-A) that could contribute to the third intermediate complex in mixing experiments. The B-30 supershift also provides greater resolution of the three receptor–DNA complexes.

The in vitro mixing experiment shown in Figure 6 was performed under conditions similar to that of Figure 5, except the total time of the assay was substantially reduced. Thus, the formation of a third intermediate DNA complex is not likely due to exchange of ligands during in vitro mixing of PR-A and PR-B. In further support of this, only one intermediate mobility complex is obtained after mixing of PR-A–RU486 with PR-B–R5020. If ligand exchange were occuring, we would expect to see the formation of multiple intermediate complexes since RU486 alters the mobility of PR–DNA complexes compared with R5020.

To determine whether heterodimer formation between PR–R5020 and PR–RU486 is unique to baculovirus-expressed receptors, or could also occur with endogenous PR from mammalian cells, A and B receptors from T47D cells were separated by gradient centrifugation by the B-30 MAb as in Figure 3, and then mixed in vitro prior to binding to DNA. As shown in Figure 7A, gradient peaks 1 and 2, to which no MAb is added (Figure 7A, left panel), generate three PR–DNA complexes corresponding to BB, and AB, and AA dimers (Figure 7B). Separated PR-B (after addition of B-30 MAb) bound to either RU486 or R5020 (peaks 3 and 4), produces a single DNA complex as does separated PR-A bound to RU486 or R5020 (peaks 5 and 6) (Figure 7B). It should be noted that PR-B is bound to the B-30 MAb, and thus the PR-B–DNA complexes are supershifted. Also, in all cases, receptor dimers exhibit faster electrophoretic mobility when bound to RU486, even when supershifted with the B-30 MAb. Separated A and B receptors were next mixed in various combinations in vitro at 0–4 °C prior to gel mobility shift assay. Mixing of PR-A and PR-B, when each is bound to the same ligand (R5020 or RU486), results in the appearance of a third intermediate mobility complex (Figure 7C, mixed peaks 3 + 5 and 4 + 6). When PR-B–R5020 is mixed with PR-A–RU486, a third complex of intermediate mobility is also formed (Figure 7C, mixed peaks 4 + 5). Mixing in the opposite direction produces similar results; PR-B–RU486 mixed with PR-A–R5020 yields a third complex indicative of PR-A·PR-B heterodimers (Figure 7C, mixed peaks 3 + 6). As with baculovirus-expressed receptors, less of the intermediate DNA complex is formed when the two PR isoforms are bound to opposite ligands compared with mixing of PR isoforms bound to the same ligand. The results in Figure 7 indicate that

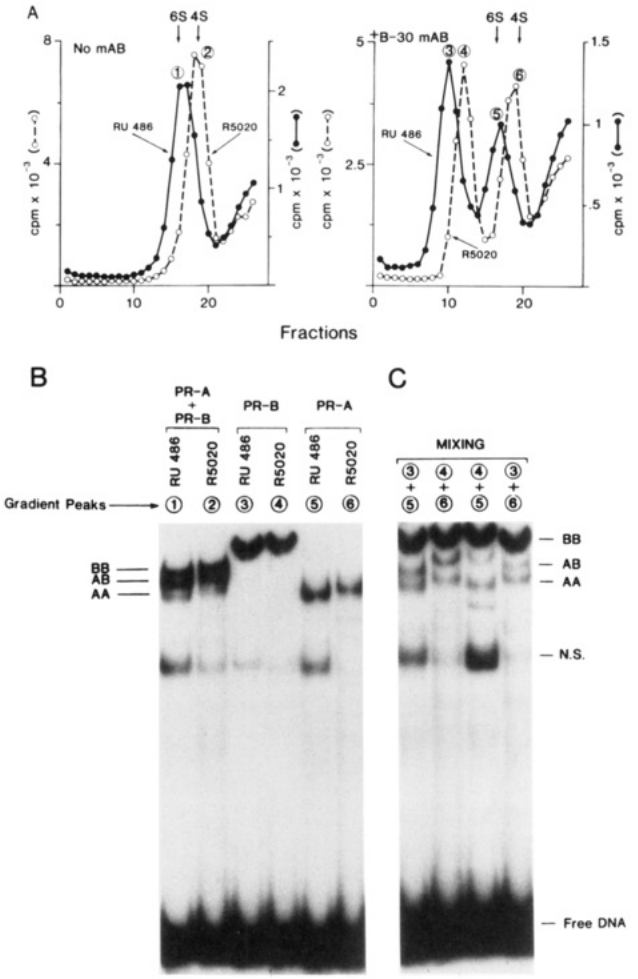

FIGURE 7: Detection of R5020·RU486 PR heterodimers bound to DNA with endogenous T47D receptors. (A) PR from T47D cells were bound to [^{3}H]RU486 or [^{3}H]R5020 as described in Figure 3A, incubated with or without B-30 MAb, and centrifuged on sucrose gradients to separate PR-B from PR-A. (B) Aliquots of peak fractions collected from the gradients (numbered 1–6) were analyzed for binding to ^{32}P-labeled PRE by gel mobility shift assay. The DNA binding reaction was for 1 h at 0 °C. (C) Separated PR-A and PR-B (peaks 3–6) were mixed as indicated in ratios of 1:1 (v/v) for 30 min at 0 °C. Aliquots (5 μL) of the mixture were then analyzed by the gel mobility shift assay.

endogenous PR behave in a manner similar to that of baculovirus-expressed receptors in their ability to form PR–RU486·PR–R5020 heterodimers that bind to a PRE.

DISCUSSION

The present results show that RU486 does not impair progesterone receptor dimerization in the absence of DNA. Paradoxically, RU486 promotes the steps of dimerization and binding to a PRE more efficiently than the agonist R5020, yet largely fails to induce PR-mediated transcriptional activation in vivo (El-Ashry et al., 1989; Meyer et al., 1990). Enhanced PR dimerization induced by RU486 was also reported recently by Skafar (1992) in studies where the cooperativity of ligand binding was used to measure a dimerization constant for receptors (which reflects the receptor concentration required for dimerization). It was shown that calf uterine PR bound to RU486 has a significantly lower dimerization constant (2.57 nM) than PR bound to progesterone (7 nM). The differences observed in the present study for dimerization and DNA binding do not appear to be minor. RU486 promotes a 2-fold increase over R5020 in measurable solution PR dimers and also confers a degree of stability of PR dimers to salt dissociation (Figure 1). RU486 also induces a 2–3-fold higher DNA binding activity over that induced by R5020 (Figure 2). In a previous study, we examined the kinetics of PR dissociation from a PRE in vitro and found that receptors bound to RU486 displayed an off-rate that was 2-fold slower when compared to PR bound to R5020 (El-Ashry et al., 1989). These differences in properties of PR are likely due to an alteration in the PR molecule itself and not to differences in kinetics of ligand binding, since RU486 and R5020 have been shown to have similar binding affinities for human PR (Horwitz, 1985).

At present, it is unclear whether or how enhanced PR dimerization contributes to the in vivo antagonist activity of RU486. Some transcription factors, that use dimerization as a mechanism to control DNA binding, readily exchange their dimer subunits (Shuman et al., 1990), whereas others are not freely exchangeable (Manak & Prywes, 1991). Thus, dissociation, or exchange, of dimer subunits could be one mechanism whereby DNA-bound transactivators subsequently interact with other nuclear factors. PR apparently falls in the category of a dimeric factor that can readily undergo subunit exchange. This is supported by the findings that separated PR-A and PR-B rapidly form PR-A·PR-B dimers upon mixing in vitro (Figures 5–7) and that dimers formed in solution readily dissociate during sucrose gradient centrifugation (Figure 3). Glucocorticoid receptors also appear to be in this category since chemical cross-linking is required for the maintenance of dimers during gradient centrifugation (Wrange et al., 1989). Ligand-activated estrogen receptors, by contrast, do form more stable 5 S dimers that are distinguishable on density gradients from 4 S monomers (Jensen, 1991; Linstedt et al., 1986; Notides et al., 1981). A direct comparison of the calf uterine estrogen receptor (ER) and progesterone receptor has indeed confirmed that ER have a much lower dimerization constant than PR. Dimerization constants of approximately 7 and 0.3 nM, respectively, were reported for PR and ER, indicating that ER is dimeric at a 20-fold lower receptor concentration than PR (Skafar, 1991).

How might a more stable dimer affect transcriptional enhancement? A number of observations indicate that interactions among factors in the transcription complex are specific but of relatively low affinity (Frankel & Kim, 1991) and an abnormally tight interaction could conceivably interfere with the function of multiple activators in combination. In this light, it is important that PR bound to RU486 are completely inactive in stimulating a reporter gene driven by PREs in the complex MMTV promoter (El-Ashry et al., 1989; Meyer et al., 1990), yet exhibit at least partial agonist activity when PREs are present in less complex, synthetic promoters (Bagchi et al., 1990; Klein-Hitpass et al., 1991; Meyer et al., 1990). We therefore propose as one possibility that alterations in the kinetics of subunit exchange of PR dimers could contribute to the inability of receptors to interact efficiently with other nuclear factors required for transcriptional enhancement.

An additional or alternative mechanism for the action of RU486 is the likelihood that RU486 induces a conformation in the PR molecule that is distinct from that induced by progestin agonists. We propose that the enhancement in solution dimerization of PR induced by RU486 is the consequence of an altered conformation and that this alteration may be central to contributing to the biological effects of RU486. Earlier studies showed that transformed PR bound to RU486 either in vivo (Mullick & Katzenellenbogen, 1986; El-Ashry et al., 1989) or in vitro (El-Ashry et al., 1989)

sediments at 6 S on density gradients whereas transformed PR bound to R5020 and unliganded PR stripped free of heat shock proteins by salt (Bagchi et al., 1990) both sediment at 4 S. At the time of these observations, the mechanism responsible for the faster 6 S sedimentation was not known, but one speculation was an enhanced receptor–receptor interaction (Mullick & Katzenellenbogen, 1986). The results in Figures 1 and 2, showing that RU486 enhances solution dimerization of PR, prompted us to examine this possibility. However, we found that RU486 does not stabilize PR-A·PR-B heterodimers against dissociation during the gradient centrifugation (Figure 3). RU486 interaction also causes recombinant PR, synthesized in a heterologous baculovirus system, to sediment at 6 S instead of the 4 S position of recombinant PR bound to R5020; this suggests that cell-specific factors are not involved in this effect of RU486. Another possible explanation for the 6 S sedimentation is that activated receptors bound to RU486 may dissociate to a different extent from receptor-associated heat shock protein 70 (hsp 70). Activated PR bound to progestin agonists dissociate from hsp 90, but remain associated with some hsp 70 (Kost et al., 1989; Oñate et al., 1991). As assessed by coimmunoabsorption of hsp 70 with receptor-specific antibodies, we have detected no obvious difference between activated receptors bound to R5020 or RU486 with respect to the relative amounts of hsp 70 that remain associated with PR (P. A. Estes and D. P. Edwards, unpublished results). These results taken together are consistent with the conclusion that RU486 binding directly alters the conformation of PR to cause the faster 6 S sedimentation on gradients. Another observation that supports the conclusion that RU486 induces a distinct conformation in PR is the faster mobility of PR–DNA complexes in the gel mobility shift assay. This was first shown by El-Ashry et al. (1989), and later confirmed by others (Meyer et al., 1990; Klein-Hitpass et al., 1991). We now provide evidence that this alteration is due to a direct interaction of RU486 with PR since this effect occurs whether RU486 is added to T47D PR in vitro (Figure 2) or in vivo, or with crude (Figure 4) or highly purified recombinant PR expressed in a baculovirus system (S. A. Oñate, S. K. Nordeen, and D. P. Edwards, unpublished results).

The increased *s* value and faster electrophoretic mobility on nondenaturing gels at first appear to be contradictory; however, these observations can be reconciled if RU486 were to induce an abnormally dense or spherical shape in the receptor compared with receptors bound to a progestin agonist. Recent studies with a site-directed MAb (C262) to the extreme C-terminus of PR provide independent evidence to support the concept that PR bound to RU486 are in a different conformation than PR bound to R5020 and further that the C-terminus appears to be involved in this difference. We have shown that the C-terminal epitope recognized by this MAb is accessible when PR are bound with RU486 yet is completely occluded when receptors are bound to progesterone or R5020 (Weigel et al., 1992). Others have similarly reported structural differences when PR are bound to RU486 or progestin agonists, indicating that RU486 binds to PR differently and/or induces a distinct conformation (Geier et al., 1987; Skafar, 1991b). Several estrogen antagonists, that do not interfere with binding of estrogen receptors to specific DNA sites, have also been shown to induce alterations in the mobility of receptors bound to specific DNA (Fawell et al., 1990; Green, 1990; Kumar & Chambon, 1988; Pham et al., 1991), suggesting that antiestrogens induce a distinct conformation in ER. Thus, a common mode of action for steroid antagonists may be to induce a distinct and aberrant conformational change in the receptor dimer bound to DNA.

Because RU486 binding to PR appears to induce a conformation distinct from that of R5020, and most likely involves the C-terminus (Weigel et al., 1992) which harbors dimerization sequences (Fawell et al., 1990; Chambon & Kumar, 1988), we sought to determine whether a receptor subunit bound with RU486 was capable of recognizing and forming a heterodimer with another subunit bound with R5020. On the basis of results of the coimmunoprecipitation assay in Figure 5, this conformational change does not appear to be sufficient to impair solution heterodimerization between PR bound to R5020 and PR bound to RU486. PR–RU486·PR–R5020 heterodimers formed in solution also possess the ability to recognize and bind to a PRE in gel-shift assays, although to a lesser extent than receptor dimers where both subunits are bound with the same ligand (Figure 6). In a similar study, Meyer et al. (1990) reported that in vitro mixing of one human PR isoform liganded with RU486 (PR-A) and another liganded with R5020 (PR-B) failed to produce an intermediate heterodimer complex bound to a PRE. They also showed by transient cotransfection of human PR bound with RU486 and chick PR bound with R5020 (chick PR does not bind RU486 and thus precludes ligand exchange in vivo as an interpretation of results) that excess RU486 in vivo antagonized the transcriptional activity of chick PR for a PRE–CAT reporter gene induced by R5020. Because Meyer et al. (1990) were unable to detect PR–RU486·PR–R5020 heterodimers bound to a PRE in vitro, it was concluded that PR–RU486 dimerization interfaces are not compatible for heterodimerization with PR–R5020. Therefore, dominant negative effects through heterodimerization were ruled out as the mechanism responsible for observed in vivo effects of RU486 on chick PR. However, the ability of RU486- and R5020-liganded receptors to form dimers in solution was not examined in these studies (Meyer et al., 1990). On the basis of the results of the present solution dimerization experiment (Figure 5), we submit that a dominant negative effect in vivo cannot be ruled out. It is possible that heterodimerization may occur efficiently in solution, while the ability of RU486·R5020 heterodimers to bind to specific DNA may be dependent upon the nature of the PRE. Our study employed a partial palindromic oligonucleotide corresponding to the distalmost PRE/GRE of the natural MMTV promoter, whereas Meyer et al. (1990) used a synthetic perfect palindromic PRE. If PR–R5020·PR–RU486 heterodimers are asymmetric, they may bind less efficiently to a perfect palindrome. In this respect, it is relevant that no perfect palindromes have been reported as progestin or glucocorticoid response elements in natural promoters (Beato, 1989). It should be noted that a dominant negative effect in vivo could be elicited either through heterodimerization occurring prior to DNA binding (i.e., in solution) or upon DNA binding.

The fact that PR bound to RU486 and R5020 are capable of forming heterodimers in vitro (both in solution and at least to some extent as heterodimers bound to an imperfect PRE) may aid in the interpretation of earlier biologic results with RU486. We previously reported that PR-mediated gene transcription, induced by R5020 in T47D cells (using a stably transfected MMTV–CAT reporter gene), was completely blocked by a dose of RU486 that was one-fifth that of R5020 (El-Ashry et al., 1989). Since the two ligands have approximately equal affinity for the human PR (Horwitz, 1985), RU486 behaved as a more potent antagonist in vivo than expected by simple competition of RU486 for progestin binding

sites on the PR. One possible mechanism to explain these biological results is that receptors bound to RU486 could inactivate receptors bound to R5020 by heterodimerization; the implication is that not all receptor binding sites need to be occupied by RU486 for complete progestin antagonism to occur. Further experimentation will be required to determine whether such heterodimerization occurs to a significant extent in vivo and whether or not the proposed heterodimers are functional.

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Registry No. RU486, 84371-65-3; progesterone, 57-83-0.