Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 72–76, January 1991
Biochemistry

Dimerization of mammalian progesterone receptors occurs in the absence of DNA and is related to the release of the 90-kDa heat shock protein

(steroid receptors/dimers/DNA-binding protein/progesterone response elements)

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Communicated by Elwood V. Jensen, September 21, 1990

ABSTRACT **In this study we have demonstrated that dimerization of mammalian progesterone receptors (PR) occurs in the absence of DNA. A specific immune coisolation assay was performed on extracts of T-47D human breast cancer cells with a monoclonal antibody specific for the full-length B form of progesterone receptor (PR-B). This resulted in coisolation of significant amounts of truncated form-A receptors (PR-A), indicating the presence of stable PR-A·PR-B dimers in solution. A positive correlation was observed between the ability of different receptor forms to oligomerize in solution and their ability to bind to specific DNA sequences. The ability to form stable PR-A·PR-B oligomers in the absence of DNA was also found to correlate with release of 90-kDa heat shock protein (hsp90) from the unactivated PR complex. These results support the hypothesis that dimerization in the absence of DNA is an important mechanism controlling receptor DNA-binding function and that hsp90 release may be a key step regulating dimerization. This suggests that hsp90 may function to repress DNA-binding activity indirectly by blocking receptor dimerization.**

Steroid receptors are members of a superfamily of ligand-dependent transcriptional regulatory proteins that bind to hormone response elements (HREs) of target genes (1). HREs often exhibit a palindromic character and various footprinting techniques have shown a dyad symmetry for receptor contact (2, 3), suggesting that steroid receptors bind to DNA as dimers. More-direct evidence for this has been provided by studies indicating a stoichiometry of two receptor molecules bound to one HRE unit (2, 4). Additionally, gel mobility-shift assays performed with receptor size variants of two different lengths have been reported to generate three mobility classes of DNA complexes consistent with estrogen receptors (ER) (5, 6), glucocorticoid receptors (GR) (2), and progesterone receptors (PR) (7) all existing as dimers when complexed to their cognate HREs. Whether receptor dimerization occurs in solution as a regulatory step to control DNA-binding function, as has been described for other transcriptional regulatory proteins (8, 9), remains relatively unexplored.

Several different classes of receptors can form an association with the 90-kDa heat shock protein (hsp90) in an 8–10S non-DNA-binding complex prior to their "activation" by hormone (10, 11). Activation of receptors, either in cell-free cytosol or in intact cells, results in dissociation of hsp90 and receptor conversion to a 4S DNA-binding form (10, 11). Thus, receptor-associated hsp90 appears to repress DNA binding activity in the absence of hormone. Consequently, one step in the process of receptor activation is thought to be hormone-induced dissociation of hsp90. The mechanism by which hsp90 represses DNA-binding activity is not known, however.

Human PR in T-47D human breast cancer cells are expressed as two hormone-binding proteins of ≈94 kDa and ≈120 kDa, termed PR-A and PR-B, respectively (12). PR-A is a truncated form of PR-B, missing N-terminal amino acids, but is otherwise identical to PR-B in the DNA- and steroid-binding domains (13). In the present study we have used an immune coisolation assay, with a monoclonal antibody (mAb) specific for PR-B, to attempt to detect PR-A·PR-B oligomers and to examine directly whether PR is capable of dimerizing in the absence of DNA. This specific immune coisolation assay was also used to explore the possibility that hsp90 may act to inhibit DNA binding indirectly by blocking dimerization of receptors.

MATERIALS AND METHODS

Materials. [^{3}H]R5020 (promegestone; 17α,21-dimethyl-19-norpregna-4,9-diene-3,20-one, [*17α-methyl-*^{3}H], 87 Ci/mmol; 1 Ci = 37 GBq) and unlabeled R5020 were obtained from DuPont/NEN products. mAbs AB-52 and B-30 (both mouse IgG1) were prepared against purified human PR as described (14). AB-52 recognizes both PR-A and PR-B, whereas B-30 recognizes only PR-B. mAb AC-88 (mouse IgG1) was prepared against hsp90 by Riehl *et al.* (15).

Cell Culture and Receptor Preparations. T-47D human breast cancer cells were cultured as described (7, 14). Buffers used for extraction of PR were derived from TEG [10 mM Tris base, adjusted with HCl to pH 7.4/1 mM EDTA/10% (vol/vol) glycerol]. T-47D cells were homogenized in TEDG (TEG containing 1 mM dithiothreitol) containing a mixture of protease inhibitors (14). Cytosolic PR from non-hormone-treated cells were prepared as a 105,000 × *g* supernatant of homogenates. To stabilize the unactivated 8–10S (actual value is 9 S) cytosolic receptor complex, 20 mM sodium molybdate was included in homogenization buffers. To activate cytosolic PR *in vitro* to the 4S form, cytosols prepared in TEDG were incubated with 20 nM R5020 for 4 hr at 4°C followed by exposure to 0.5 M NaCl for another 1 hr at 4°C. Nuclear 4S PR activated by incubation of intact cells for 1 hr at 37°C with 20 mM R5020 was prepared as a 0.5 M NaCl extract of isolated nuclei (7, 16). The sedimentation rates on sucrose density gradients for each of these PR forms have been published (7). Activated receptors were dialyzed at 4°C against TEDG buffer to reduce the salt concentration prior to immune isolation and DNA-binding assays. The number of

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Abbreviations: PR, progesterone receptor(s); ER, estrogen receptor(s); GR, glucocorticoid receptor(s); PRE, progesterone response element; hsp, heat shock protein; mAb, monoclonal antibody; HRE, hormone response element.

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receptor steroid-binding sites in cell extracts was determined by use of dextran-coated charcoal (7, 14).

Immune Isolation of Human PR. Protein A-Sepharose (Repligen, Cambridge, MA) was coated noncovalently with mAbs by first binding a secondary bridging rabbit anti-mouse IgG (Cappel Laboratories) to the resins, followed by incubation with mAb at a concentration of 10 μg/100 μl of protein A-Sepharose suspension. Resins were then washed with TEG (pH 7.4) by centrifugation to remove unbound mAb and incubated with aliquots of cytosolic or nuclear PR for 4 hr at 4°C on an end-over-end rotator. After absorption of receptors, protein A-Sepharose was washed three times by centrifugation (wash buffers are indicated in figure legends) followed by transfer to a clean tube and one additional wash.

SDS/PAGE and Western Immunoblotting. Immobilized PR complexes were eluted from protein A-Sepharose with SDS sample buffer. Electrophoresis and Western immunoblotting of extracted PR (7, 14) and hsp90 (15) were carried out as described, using [^{35}S]protein A (Amersham) and autoradiography as the detection method. Dried nitrocellulose blots were scanned directly for ^{35}S in protein bands with a Bioscan 200 Image Scanner (Bioscan, Washington, DC).

Gel Mobility-Shift Assays. PR in cytosol or nuclear extracts were incubated with a ^{32}P-end-labeled, double-stranded synthetic oligonucleotide (5′-gatcTTTGGTTACAAACTGTTCT-TAAAACGAG-3′ base-paired with 3′-AAACCAATGTT-TGACAAGAATTTTGCTCctag-5′) that corresponds to the most distal (base pairs −189 to −162 from the transcription start site) HRE of mouse mammary tumor virus. This element is responsive to both glucocorticoids and progestins (17) and will be referred to as a progesterone response element (PRE). The DNA binding buffers and conditions for gel electrophoresis and autoradiography have been described (7).

RESULTS

Oligomerization of Activated PR in the Absence of DNA. We were interested in determining whether PR dimers were formed in solution in the absence of target DNA. We reasoned that if the A and B forms of PR were to randomly associate in solution prior to binding DNA, then immune isolation of T-47D cell extracts with a PR-B-specific mAb would result in coisolation of PR-A. The different forms of native receptors indicated in Fig. 1 were immune isolated with either AB-52 (PR-A- and PR-B-specific), B-30 (PR-B-specific), or a control mAb. Each of the isolated complexes was then analyzed by Western immunoblotting with the AB-52 mAb, which recognizes both the A and the B form of the receptor. Immune isolations of both cytosolic and nuclear forms of activated PR with B-30 resulted in coisolation of significant amounts of PR-A (Fig. 1*B*), indicating the presence of PR-A·PR-B oligomers that are stable to immune isolation. Parallel immune isolations performed with an unrelated control mAb (Fig. 1*C*) showed that coisolated PR-A resulted from a specific association with PR-B.

The Western blot data in Fig. 1*B* (and from replicate experiments) were quantified by direct scanning of the nitrocellulose filters to determine the relative amounts of PR-A present in the B-30-isolated complex (Table 1). From this analysis it appears that unactivated cytosolic PR have little or no ability to form PR-A·PR-B oligomers in solution, since only trace amounts of PR-A (2% of the total PR-B) were detectable. By contrast, PR-A represented 10% and 23% of the total immune isolated PR-B obtained from *in vitro* activated cytosolic and "*in vivo*" activated nuclear PR, respectively (Table 1). If one assumes that PR-A and PR-B are expressed in equal amounts, binomial distribution predicts the existence of AA, AB, and BB dimers in the ratio of 1:2:1, respectively. Therefore, since the B-specific mAb will isolate both AB and BB dimers, the maximal theoretical amount of

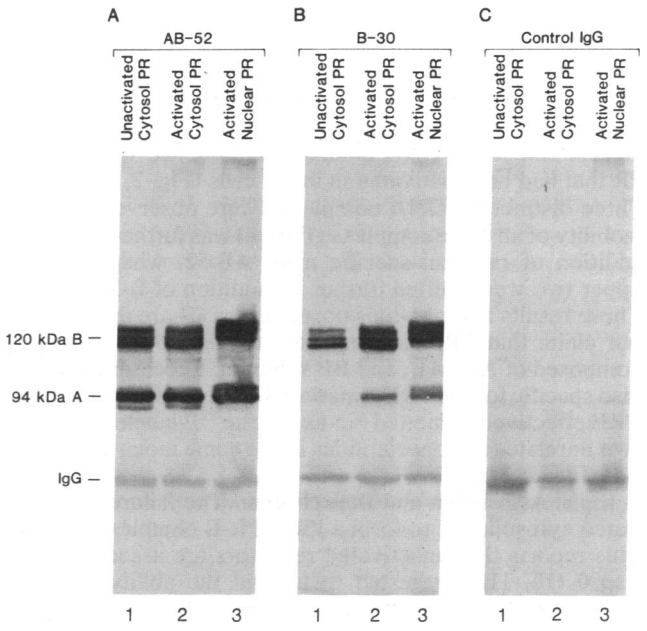

FIG. 1. Detection of PR-A·PR-B oligomers in solution by immune coisolation of PR-A receptors with a mAb specific for PR-B (B-30). Equal amounts (8 pmol based on steroid-binding assay) of the three native forms of PR indicated were immune isolated with mAb AB-52 (*A*), mAb B-30 (*B*), or an unrelated control IgG mAb (*C*). PR·mAb complexes were adsorbed to protein A-Sepharose, the resins were then washed by centrifugation (in TEG buffer for activated PR and TEG plus 20 mM sodium molybdate for unactivated PR), and the immobilized PR complexes were then extracted for Western blot assay with AB-52. Apparent molecular masses were determined by use of prestained molecular weight markers (Bethesda Research Laboratories).

PR-A that can be coisolated using this assay is half of the total isolated PR-B. Since the maximal percent of coisolated PR-A obtained experimentally was 23% with activated nuclear PR, it appears that only half of activated receptors are capable of forming stable oligomers in the absence of DNA.

Parallel immune isolations performed with the AB-52 mAb as a control for total cellular PR (Fig. 1*A*) illustrate that PR-A and PR-B are expressed in T-47D cells in approximately equal amounts. Also shown in Fig. 1*A* is an apparent increase in molecular mass of activated nuclear PR in SDS/polyacrylamide gels, which has been demonstrated (18) to be a distinctive feature of hormone-dependent phosphorylation of PR in T-47D cells. This characteristically results in a decrease in mobility of all PR-B (PR-B migrates as a triplet before and after hormone) but only a portion of PR-A (PR-A is primarily a single band in the absence of hormone). This results in PR-A splitting into a doublet (Fig. 1*A*, lane 3). Activated PR bound with hormone in cell-free cytosol, which displays a lower ability to oligomerize in solution (Table 1), fails to exhibit this phosphorylation-induced increase in apparent molecular mass (Fig. 1*A*, lane 2).

Table 1. Quantification of PR-A·PR-B oligomerization

PR form	PR-A/PR-B ratio, mean ± SEM
Unactivated cytosolic	2 ± 0.6% (n = 7)
Activated cytosolic	10 ± 1.2% (n = 11)
Activated nuclear	23 ± 2.7% (n = 9)

The PR-A/PR-B ratio represents the relative amounts of PR-A that were coisolated with the PR-B-specific mAb, expressed as a percent of total PR-B. Values were obtained by direct scanning (Bioscan) of dried Western blot nitrocellulose filters for radioactivity (bound [^{35}S]protein A) in PR-A and PR-B bands.

The same cytosolic and nuclear PR preparations used for immune coisolation were also assayed by gel mobility shift for DNA-binding activities (Fig. 2). Unactivated cytosolic PR failed to bind to the ^{32}P-end-labeled PRE. *In vitro* activated cytosolic PR exhibited low but detectable binding, while the highest amount of DNA binding was obtained with nuclear PR that had been activated in intact cells (Fig. 2, lanes 1–3). Three distinct PR·DNA complexes were observed, and the mobility of all three complexes (lane 4) was further shifted by addition of receptor-specific mAb AB-52, while only the upper two were shifted further by addition of B-30 (lane 5). These results and previous observations (7) are the basis for our claim that PR are complexed to PRE DNA as dimers composed of AA, AB, and BB subunits. Receptor binding is also specific for the PRE, since a 500-fold excess of unlabeled PRE effectively inhibited binding to the ^{32}P-labeled PRE, yet two unrelated oligonucleotides in the same molar excess did not (Fig. 2, lanes 7–9).

hsp90 Association and Dimerization. The failure of unactivated cytosolic PR to form a PR-A·PR-B complex (Fig. 1*B*), plus reports that unactivated receptors are associated with hsp90 (10, 11), suggested to us that the ability of PR to oligomerize in solution might depend upon the presence or absence of hsp90. To examine this further, receptors were prepared with various amounts of associated hsp90 and were then assayed for their ability to assemble into PR-A·PR-B oligomers in the absence of DNA. The specific immune coisolation assay was performed as above, except that B-30-isolated complexes were analyzed by Western immunoblot for the presence of both PR-A (mAb AB-52) and hsp90 (mAb AC-88). An inverse relationship was observed between the presence of hsp90 in the B-30-isolated complex and the extent

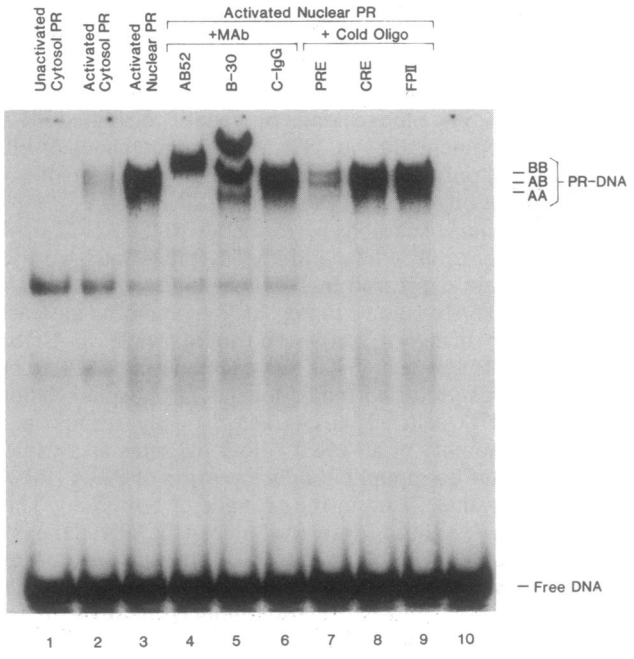

FIG. 2. Binding of receptors to PRE DNA as detected by gel mobility-shift assay. Aliquots of cytosol or nuclear extracts containing 0.06 pmol of PR (based on steroid-binding assay) were incubated with 0.1 ng of a ^{32}P-end-labeled PRE oligonucleotide and reactants were submitted to electrophoresis in nondenaturing polyacrylamide gels as described (7). Arrows indicate positions of the PR·DNA complexes believed to contain AA, AB, and BB dimers. Lanes 1–3, different forms of PR; lanes 4–6, addition of receptor-specific mAb AB-52 or B-30 or an unrelated control mAb (C-IgG) to activated nuclear PR; lanes 7–9, addition of a 500-fold molar excess of unlabeled ("cold") PRE or unrelated oligonucleotides, cAMP response element (CRE) and FPII (19), to activated nuclear PR; lane 10, no added cellular protein.

of PR-A association with PR-B (Fig. 3*A*). Molybdate-stabilized cytosolic PR contained high amounts of hsp90 and no detectable PR-A·PR-B oligomers, while treatments that lead to a progressive dissociation of hsp90 from receptors resulted in a parallel increase in PR-A·PR-B oligomerization. When each of the same PR samples was analyzed by gel mobility-shift assay (Fig. 3*B*), DNA-binding activities were found to be directly correlated with the ability of each receptor preparation to oligomerize in solution (compare Fig. 3 *A* and *B*). These Western blot (Fig. 3*A*) and gel shift (Fig. 3*B*) data were quantified by direct scanning. Graphical analysis illustrates that the ability of PR to oligomerize in solution and to bind to DNA are directly related (compare Fig. 3*C Upper* and *Lower*) and that both of these activities are inversely related to the presence of hsp90 (*Lower*, •). For cytosolic PR preparations these correlations are roughly proportional and hold particularly well. However, activated nuclear PR exhibits higher activities for DNA binding and oligomerization, over that of salt-activated cytosol PR, than can be accounted for in total by further dissociation of hsp90. Although the amounts of hsp90 associated with these two activated PR forms are nearly equivalent and quite low, activated nuclear PR exhibits a 5.4-fold (average of 7-fold from multiple experiments; data not shown) higher DNA binding activity and a 2.4-fold (average of 2.3-fold from multiple experiments, Table 1) higher ability to oligomerize. This suggests that dissociation of hsp90 from receptors is necessary but not sufficient for maximal activation of oligomerization and DNA-binding functions.

Influence of Hormone-Dependent Phosphorylation on Stability of PR Oligomers in Solution. A possible mechanism to explain the higher ability of activated nuclear PR to oligomerize that cannot be accounted for in total by hsp90 release was suggested by the fact that only this form of PR exhibited hormone-dependent phosphorylation (Fig. 1*A*). To examine this further, we analyzed the stability of activated nuclear PR-A·PR-B oligomers against salt dissociation, by performing the immune coisolation assay with various amounts of NaCl. Fig. 4 shows that in the absence of NaCl, the PR-A that was coisolated with the PR-B-specific mAb B-30 consisted of both PR-A phosphoforms: the underphosphorylated form (designated PR-A2) that is present predominantly in the absence of hormone and the higher phosphoform (PR-A1) generated predominantly in response to hormone binding in intact cells (see Fig. 1*A*). With increasing concentrations of NaCl the amount of total PR-A that remained associated with PR-B was decreased, but dissociation was preferential for the less phosphorylated form of PR-A (PR-A2). Only the slower-mobility PR-A form that had undergone hormone-induced phosphorylation (PR-A1) remained associated with PR-B at the higher salt concentrations. This suggests that hormone-dependent phosphorylation may impart greater stability to PR dimers in solution.

DISCUSSION

The present study has demonstrated that human progesterone receptors are capable of oligomerizing in solution in the absence of DNA. That truncated PR-A can be coisolated with a mAb that recognizes only full-length PR-B provides direct evidence that PR-A and PR-B are capable of forming a stable heterocomplex in solution. By this method, however, it was not possible to conclusively determine whether the heterocomplex was composed of dimers or higher-order multimers. Since gel mobility-shift assays generated three specific PR-DNA complexes (Fig. 2), consistent with PR existing as a dimer when complexed to DNA (7), we strongly suspect that the PR-A·PR-B oligomers detected in solution represent heterodimers composed of one subunit of PR-A and one of PR-B. These findings suggest that PR may be capable of

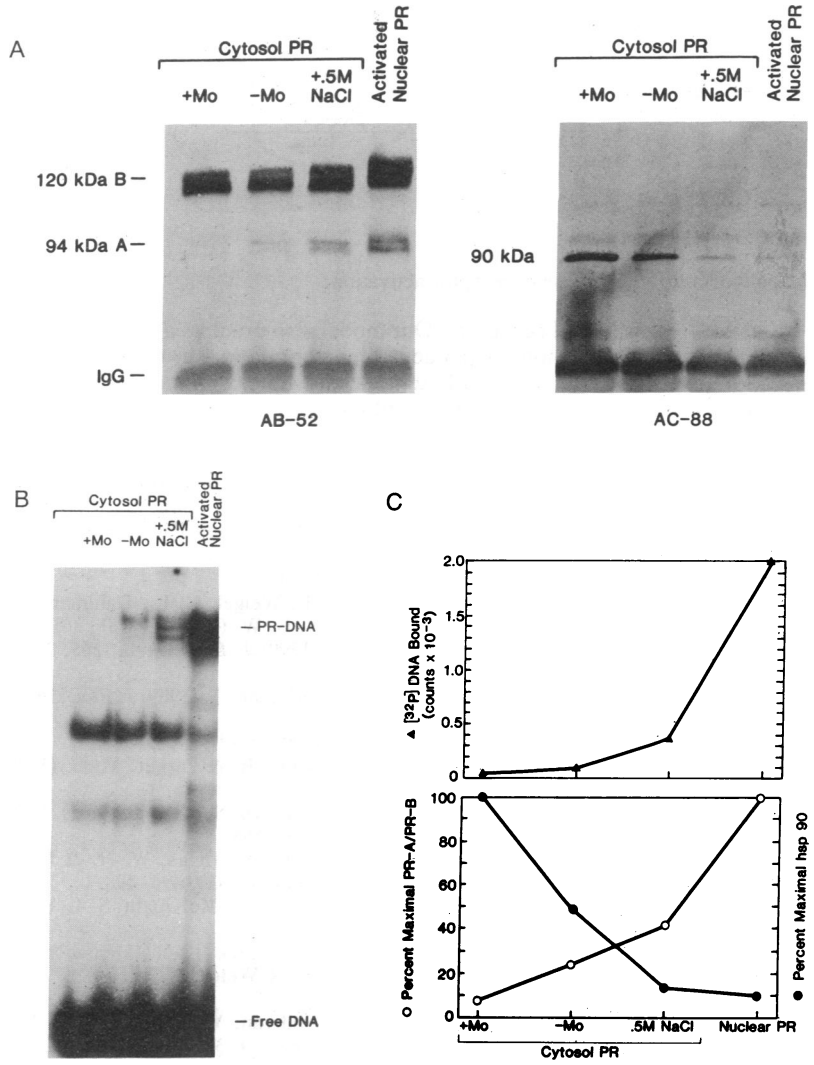

FIG. 3. Dissociation of hsp90 is correlated with formation of PR-A·PR-B oligomers in solution and with PR binding to DNA. (*A*) Cytosolic PR from non-hormone-treated cells were prepared in either TEDG buffer containing 20 mM sodium molybdate (+Mo), in TEDG alone (−Mo), or in TEDG after which PR were incubated for 4 hr at 4°C with 20 mM R5020 followed by addition of 0.5 M NaCl and incubation for another 1 hr at 4°C. Activated nuclear PR were prepared as a salt extract of isolated nuclei after incubation of intact cells with R5020 for 1 hr at 37°C. Equal amounts (8 pmol based on steroid-binding assay) of each PR preparation were immune isolated with B-30, as in Fig. 1, except that the isolated complex preparation was divided in half for Western blot assay with AB-52 for detection of PR-B and PR-A (*Left*) or with AC-88 for detection of hsp90 (*Right*). (*B*) Gel mobility-shift assay of the same cytosolic and nuclear PR preparations. (*C*) Western blots (*A*) and the mobility-shift gel (*B*) were directly scanned to quantitate the relative amounts of PR-A and hsp90 associated with immune-isolated PR-B complexes and DNA-binding activities. As a measure of the extent of PR oligomerization the ratio of PR-A to PR-B (○) in each lane of *A Left* was determined and expressed as the percent maximum ratio of PR-A to PR-B obtained by B-30 immune isolation of activated nuclear PR. The amount of hsp90 (●) in each lane of *A Right* was expressed as the percent maximum contained in the molybdate-stabilized PR complex. DNA bound by each PR form was expressed as total counts of [^{32}P]DNA bound (▲).

forming both homodimers (AA or BB) and heterodimers (AB) as a mechanism to create receptor molecules with different functional activities. If so, factors that control the relative amounts of PR-A and PR-B may provide a unique combinatorial regulatory mechanism for this receptor system. It is interesting that chicken PR-A and PR-B have been shown to exhibit different target-gene specificities (20), and dramatic seasonal variation in expression of PR-A in chicken oviduct has been reported (21).

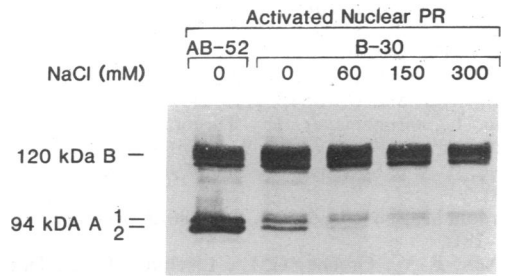

FIG. 4. Effects of salt on dissociation of PR-A·PR-B oligomers. Nuclear PR activated by hormone binding in intact cells (8 pmol) were immune isolated with AB-52 (as a control for total cellular PR) or B-30 as in Fig. 1 except that PR complexes immobilized to protein A-Sepharose were washed with TEG containing the NaCl concentrations indicated. After washing of resins, receptors were extracted and analyzed by Western blot assay with AB-52 for the presence of both PR-B and PR-A.

Indirect evidence, based on kinetic and hydrodynamic properties, that activated ER are capable of forming stable dimers in the absence of DNA has been available for some time (22, 23). More recently, immune coisolation of truncated ER with an antibody that recognizes an epitope present only in the full-length molecule has provided more direct evidence for the existence of ER dimers in the absence of DNA (6). Detection of dimers in solution has been more difficult with other classes of steroid receptors, possibly because they are inherently less stable than ER dimers. As evidence of this, purified activated GR have been reported to form homodimers in solution that are stable to gel filtration but tend to dissociate into monomers during density gradient centrifugation (4). Interestingly, maintenance of 6S GR dimers during gradient centrifugation was shown to require stabilization by either chemical crosslinking or addition of DNA (4). Other evidence for PR dimerization in the absence of DNA comes from studies on localization of nuclear translocation sequences in the rabbit PR. Consistent with oligomerization occurring prior to DNA binding, ligand-induced nuclear uptake of a translocation-defective PR mutant was found to occur when cells were cotransfected with wild-type PR (24).

The recent report by Fawell *et al.* (6) has provided the only study as yet to directly address the question of whether steroid receptor dimerization in solution is a requirement for recognition of site-specific DNA. Deletion and site-directed mutagenesis have identified a conserved region of heptad-repeat hydrophobic residues in the steroid-binding domain

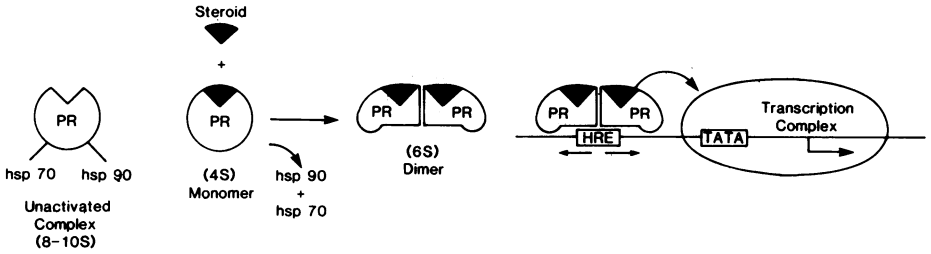

FIG. 5. Proposed multistep model for the process of receptor activation.

that is responsible for mediating dimerization of the ER in the absence of DNA. They also reported a direct correlation between the ability of ER mutants to form dimers in solution and their ability to bind to estrogen response elements. These results support the hypothesis that ER dimerization largely controls DNA-binding function. Our biochemical analyses revealing a positive correlation between the ability of different PR forms to oligomerize in solution and their ability to bind to specific DNA sites (Figs. 1–3) are also consistent with a model in which dimerization is a critical factor controlling receptor recognition of dyad symmetrical DNA.

That dissociation of hsp90 from receptors correlated both with the ability of PR to oligomerize in solution and to bind to DNA suggests that hsp90 may play a functional role in negatively controlling receptor dimerization. This raises the possibility that hsp90 may inhibit receptor–DNA binding indirectly by blocking dimerization, instead of masking the DNA-binding domain as generally presumed. Although dissociation of hsp90 may be necessary for activation of PR, we propose that this is not sufficient for maximal activation of receptor dimerization and DNA-binding functions. Evidence for this is provided by the fact that activated nuclear PR was found to be twice as efficient in forming PR-A·PR-B oligomers (Fig. 1*B* and Table 1) and to exhibit a 5- to 7-fold higher DNA-binding activity than activated cytosolic PR (Figs. 2 and 3), yet hsp90 was apparently released from both of these PR forms (Fig. 3). This suggests that hormone binding in intact cells induces modifications in receptor structure or conformation required for maximal activation that are not mimicked by hormone-binding in cell-free cytosol. Bagchi *et al.* (25) similarly concluded that dissociation of hsp90 from receptors was not sufficient for activation of PR, since addition of hormone subsequent to salt dissociation of receptors was required for receptor activation in a cell-free transcription system. We suggest that hormone-dependent phosphorylation may in part be responsible for the higher activity of nuclear PR, since nuclear PR activated by hormone binding in intact cells is exclusively modified in this fashion. PR activated in cell-free cytosol fails to exhibit hormone-induced phosphorylation (Fig. 1*A*). There is precedent for phosphorylation regulating dimerization of a gene-regulatory protein. This has been shown for the cAMP-dependent response element-binding protein, CREB (8).

Based on the results in the present study, we propose that activation of the DNA-binding function of mammalian PR is a multistep process. A working model is depicted in Fig. 5. In the absence of hormone, PR are associated with hsp90 and perhaps other proteins, including hsp70 (11, 14), to form an 8–10S unactivated complex. As an initial step in the activation process, hormone binding promotes release of hsp90, allowing 4S monomers to form a 6S dimer as an intermediate step prior to binding to DNA. The dimeric form of PR is then competent to recognize palindromic target DNA. Release of hsp90, though necessary, is not sufficient for maximal activation of receptors. Our model also predicts a functional role for hormone-dependent phosphorylation in contributing to the stability of PR dimers and thus amplifying the DNA-binding activity of receptors.

We thank Patricia A. Estes for technical assistance, Dr. Steve K. Nordeen for critical discussions, and Dr. David O. Toft for providing mAb AC-88. These studies were supported by Public Health Service Grant CA46938 and Cancer Center Core Grant P30 CA46934.

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