Progesterone Receptor Deficient in Chromatin Binding Has an Altered Cellular State*

Received for publication, September 2, 2003, and in revised form, January 22, 2004 Published, JBC Papers in Press, January 26, 2004, DOI 10.1074/jbc.M309718200

Jeannine Botos‡, Wenjuan Xian‡, David F. Smith§, and Catharine L. Smith‡¶

From the ‡Laboratory of Receptor Biology and Gene Expression, NCI, National Institutes of Health, Bethesda, Maryland 20892-5055 and the §Department of Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, Arizona 85259

Our previous work has shown that the progesterone receptor (PR) can exist in two distinct functional states in mammary adenocarcinoma cells. The differences in function included the ability to activate a promoter in organized chromatin, sensitivity to ligand, and ligand-independent activation. To determine whether these functional differences were because of altered cellular processing, we carried out biochemical analyses of the functionally distinct PRs. Although the majority of PR is localized to the nucleus, biochemical partitioning resulted in a loosely bound (cytosolic) fraction, and a tightly bound (nuclear) fraction. In the absence of progestins, the functionally distinct PRs differed significantly in partitioning between the two fractions. To characterize these fractions further, we analyzed interacting proteins of unliganded PR with chromatin by coprecipitation. We determined that PR in the cytosolic fraction associated with hsp90 and p23. In contrast, PR in the nuclear fraction consisted of complexes containing hsp90, p23, and FKBP51 as well as PR that was dimerized and highly phosphorylated. Hormone treatment significantly reduced the formation of all PR-chaperone complexes. The hsp90 inhibitor, geldanamycin, similarly blocked transcriptional activity of both functionally distinct receptors. However, the two forms of the PR differed in their ability to associate with the mouse mammary tumor virus promoter in organized chromatin. These findings provide new information about the composition and distribution of mature progesterone receptor complexes in mammary adenocarcinoma cells, and suggest that differences in receptor subcellular distribution have a significant impact on their function. These findings also reveal that transiently expressed steroid receptors may not always be processed like their endogenous counterparts.

Progesterone receptor (PR)1 functions in the development of lobular alveolar structures in the normal mammary gland (1, 2). Data from in vitro studies using cultured breast cancer cells suggest that PR exerts its effects on mammary epithelium by regulating cell cycle progression. PR positive breast cancer cells exhibit a biphasic response to progestins, initial cell division followed by long term G1 phase growth arrest (3, 4). In mammary adenocarcinoma, PR-mediated growth regulation is frequently lost. However, loss of function through receptor deletion or receptor mutation occurs only in a subset of tumors (5). Therefore, in the majority of cases, other mechanisms must be involved in loss of PR function. A better understanding of the mechanisms by which PR function can be modulated would be beneficial to developing therapy for tumors in which PR function has been lost or altered.

In vivo, steroid receptors must interact with transcriptionally inactive promoters having complex chromatin structure. Accordingly, their transcriptional activity is dependent upon a variety of proteins that modify chromatin structure, such as ATP-dependent chromatin remodelers, histone acetyltransferases, and histone deacetylases (6–9). We previously reported that the ability of the PR to activate and remodel a target promoter was conditional (10, 11). PR-mediated transcriptional activation of promoters in ordered, replicated chromatin can be studied using the mouse mammary tumor virus (MMTV) promoter. In its stably replicating form, MMTV has a highly ordered nucleoprotein structure that consists of six non-randomly positioned nucleosome families. When activated by glucocorticoids, the hormone responsive element region of the MMTV promoter undergoes a chromatin remodeling event producing a nuclease-hypersensitive site (12).

When PR is expressed through transient transfection of mouse mammary adenocarcinoma-derived cells (tPR), it cannot efficiently activate transcription from a stably replicating MMTV promoter. This, in part, is because of the fact that the tPR cannot induce chromatin remodeling (13). In contrast, the transiently expressed PR very efficiently activates a transiently transfected MMTV promoter template that has a more disorganized, accessible chromatin structure and does not require remodeling for activation (10). However, when PR is constitutively expressed through stable transfection (sPR), it acquires the ability to activate and remodel the stably replicating MMTV promoter (10), implying that PR undergoes a change in function upon continuous expression. These observations suggested that PR can exist in two functional states. Further studies reveal additional physiologically significant differences in PR function. First, tPR is dramatically more sensitive to progestins than sPR. Second, tPR is responsive to ligand-independent activation by cAMP, whereas sPR is completely refractory (11). We hypothesize that the basis of these functional differences may lie in altered cellular processing of the tPR.

Elucidating the mechanisms driving PR function as a transcriptional activator is often approached from the point of hormone binding. However, studies addressing cellular processing of...
of PR prior to hormone binding are likely to provide insight into additional mechanisms of PR action. Data from the literature support the relevance of cellular processing of PR. Studies using a cell-free system led to the discovery that PR must progress through a series of specific complexes in an ordered fashion to be competent for ligand binding (14). These complexes have been designated as early, intermediate, and mature. The early complex contains PR and hsp70, whereas the intermediate complex contains PR and hsp70, a hsp90 dimer, Hip (hsp70-interacting protein, or p48), and Hop (hsp70-organizing protein, or p60). The mature complex consists of PR, hsp90, p23, and an immunophilin (15). This cell-free system also revealed that maintenance of progestin binding of the PR required hsp70, Hip, Hop, hsp90, and p23 (16). These findings suggested a functionally significant role for PR processing in its ability to activate transcription.

In this study we have addressed the issue of altered cellular processing by comparing the composition and distribution of tPR and sPR complexes. Consistent with our previous study, we found that the unliganded PR exists in two distinct fractions based on biochemical partitioning (11). Here we have shown that there is hormone-binding competent PR in both fractions but that they contain distinct PR complexes. The distribution of the sPR and tPR between the two fractions differed significantly. However, despite its increased nuclear binding, we observed that tPR did not associate with the stably replicating MMTV promoter even upon addition of ligand. These results indicate that unliganded PR complexes are not uniform and can be differentially distributed. They also suggest that altered cellular distribution may significantly perturb PR function. Our findings are of high relevance to studies of steroid receptor function because transiently expressed receptor complexes have been designated as early, intermediate, and mature (14). These complexes progress through a series of specific complexes in an ordered fashion to be competent for ligand binding (14). These complexes have been designated as early, intermediate, and mature. The early complex contains PR and hsp70, whereas the intermediate complex contains PR and hsp70, a hsp90 dimer, Hip (hsp70-interacting protein, or p48), and Hop (hsp70-organizing protein, or p60). The mature complex consists of PR, hsp90, p23, and an immunophilin (15). This cell-free system also revealed that maintenance of progestin binding of the PR required hsp70, Hip, Hop, hsp90, and p23 (16). These findings suggested a functionally significant role for PR processing in its ability to activate transcription.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Cell Sorting—Cell line 1470.2 was derived from C127 murine mammary adenocarcinoma cells and contains multiple stably replicating copies of bovine papilloma virus-MMTV long terminal repeat-chloramphenicol acetyltransferase fusions (17). Cell line 3017.2 was derived from a single-cell clone of 1470.2 cells after stable transfection with pRSVnee, a neomycin resistance expression vector and pcPRO, a chicken PR expression vector (18). Cell line 3134 was derived from C127 cells, and it contains 200 tandemly repeated copies of stably integrated bovine papilloma virus-MMTV-Ras (19). Cell cultures were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen and Atlanta Biologicals, Norcross, GA). Transient transfections of 1470.2 cells were performed using electroporation as described previously (11). Transfected cells were plated in medium containing charcoal-stripped serum (HyClone Laboratories, Logan, UT) and incubated overnight under normal cell culture conditions. The following day, the medium was replaced and the cells were treated and harvested. For immunoprecipitation and hormone binding analysis, cells were transfected with 7 μg of pcPRO and 10 μg of pCMV-IL28, an interleukin-2 receptor substrate expression vector (HyClone Laboratories). Cells were incubated with using magnetic beads (Dynal, Great Neck, NY) coated with antibodies to the interleukin-2 receptor Tac subunit (Upstate Biotechnology, Lake Placid, NY) as previously described (11). For samples designated as R5020-treated extracts, cells were treated for 1 h at 37 °C with R5020 (PerkinElmer Life Sciences) at a final concentration of 30 μm.

Extract Preparation and Hormone Binding Analysis—Cytoplasmic and nuclear extracts were prepared as previously described (11). Briefly, 3137.2 cells and sorted, transiently transfected 1470.2 cells were suspended in HEDW buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 10 mM sodium tungstate, 2 mM dithiothreitol, and protease inhibitor mixture) and lysed by Dounce homogenization with an A pestle. Extracts were adjusted to 10% glycerol by volume (HEDGW) and centrifuged at 2000 rpm at 4 °C for 5 min. The supernatant was reserved on ice, and the nuclei were gently resuspended in HEDGW, incubated for 5 min on ice, and centrifuged as above. This supernatant was pooled with the reserved supernatant, centrifuged at 100,000 × g for 30 min at 4 °C, and denoted as the cytosolic extract. Nuclei were then extracted in HEDGW adjusted to 0.25 M NaCl for 30 min on ice, followed by centrifugation at 30,000 × g for 15 min at 4 °C. This was denoted as the nuclear extract. Cytosolic and nuclear extracts were aliquoted and stored at −80 °C until use. A volume of 5–10 μl was reserved for protein concentration measurement using the Bio-Rad protein assay reagent (Bio-Rad) as recommended by the manufacturer. For preparation of extracts from transiently transfected cells, all buffers in the extraction process contained 30 mM R5020.

To determine whether PR was associated with components of the nuclear matrix or DNA, nuclei from cells expressing sPR or tPR were extracted as reported in previous studies (21). Briefly, cells were extracted in HEDGW, 0.1% Nonidet P-40. Nuclei were aliquoted into equal volumes and extracted with either 0.25 M NaCl on ice for 30 min and/or with 500 units of RNase-free DNase (Watson) at 37 °C for 30 min. These nuclei were further extracted with 0.5% Triton X-100 for 5 min on ice. From each extraction, supernatants and the final pellets, which were resuspended in 0.1 ml of sample loading buffer, were stored for further processing.

For hormone binding analysis, 400–500 μg of extract protein from untreated cells was incubated with 4.0 × 10−9 M [3H]promegestone (R5020) (Amersham Biosciences) for one-point assays, and the following concentrations for Scatchard analysis: 4.0 × 10−9, 2.0 × 10−9, 1.0 × 10−9, 5.0 × 10−10, 2.5 × 10−10, 1.25 × 10−10, and 6.25 × 10−11 M. All assays were carried out in the absence or presence of 500-fold excess unlabeled R5020. Free R5020 was removed by exposure of extracts to dextran-coated charcoal (4% Norit-A (ICN Biochemicals, Inc., Cleveland, OH), 0.4% dextran T-70 (Sigma) in 10 mM Tris-HCl, pH 7.3). Bound R5020 was assessed by liquid scintillation counting. For one-point assays, counts per μg of protein for the cytosolic and nuclear extracts were adjusted to the percentage of total extract. Experiments were performed in triplicate. Kd values from Scatchard analysis was performed using GraphPad software (GraphPad Software, San Diego, CA).

Immunoprecipitations and Western Blotting—p23 immunoprecipitations were performed with the J3 monoclonal antibody (Affinity Bioagents, Inc., Golden, CO) as previously described (22). Briefly, Protein A-Sepharose CL-4B resin (Amersham Biosciences) was prepared according to the manufacturer’s instructions and incubated for 30 min at room temperature in 100 mM Tris-HCl, pH 8.0, with the J3 antibody. PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce). PR immunoprecipitations and heterodimerization experiments were performed with the PR22 or PR6 antibodies (Affinity BioReagents), respectively, cross-linked to Protein A-Sepharose using 20 μg dimethylsuberimidoyl chloride (Pierce)
Table I

| Cellular distribution of hormone-bound sPR and tPR |
|---------------------------------------------------|
| One-point hormone binding assays were performed as indicated under "Experimental Procedures." The values represent the percentage of total cellular hormone-bound progesterone receptor. |
| sPR | tPR |
| Cytosolic and loosely bound nuclear fraction | 84.3 ± 5.69 | 57.3 ± 7.51 |
| Tightly bound nuclear fraction | 15.7 ± 5.69 | 42.7 ± 7.51 |

provided by D. Toft (Mayo Clinic, Rochester, MN). PB6 and the FKBP51 polyclonal antibody were purchased from Affinity Bioreagents (Golden, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Luciferase and β-Galactosidase Assays—Transfected cells were harvested by scraping into 0.1 M potassium phosphate, pH 7.8, 1 mM dithiothreitol followed by repeated freeze/thaw cycles. Cell extracts were assayed for β-galactosidase and luciferase activity using a luminometer as previously reported (11).

MMTV DNA Fluorescence in Situ Hybridization (FISH) and PR Indirect Immunofluorescence—Transfection of 3134 cells was carried out as described above and plated into 6-well plates containing one coverslip per well in charcoal-stripped media. The following day, cells were treated with or without either R5020 (30 nM) or dexamethasone (100 nM) for 1 h and subjected to DNA FISH using avidin-rhodamine (Molecular Probes, Eugene, OR) as previously described (24). Following DNA FISH, cells were immunostained with PR22 (1:200), PR6 (1:200), or CREB-binding protein (CBP) antibody (1:100) for 2 h followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Molecular Probes) at 1:200 for 30 min at room temperature. Cells were viewed on a Leica DMRA microscope with a Leica 100 x 1.3 NA oil immersion objective. Images were collected with a Photometric SenSys CCD camera. The CBP antibody was purchased from Santa Cruz Biotechnology, Inc. Percent co-localization was calculated from the number of cells analyzed for each condition (n = 24–44).

RESULTS

Analysis of PR Ligand Binding in Cytosolic and Nuclear Fractions—Previous studies in this laboratory showed through immunofluorescence that the majority of the unliganded PR localized to the nucleus regardless of whether it was transiently or constitutively expressed in our mammary adenocarcinoma cell line (11). However, in both cases, the unliganded PR could be divided into two distinct biochemical fractions. One fraction was loosely associated with the nucleus and fractionated at low ionic strength with the cytosol. The other was tightly bound to the nucleus and fractionated at high ionic strength with the nuclear extract. Immunoblots of fractionated extracts indicated that, in the absence of ligand, the tPR was enriched in the nuclear fraction, whereas the sPR was predominantly cytosolic (11). In the present study, we performed one-point hormone binding assays with an excess of the progesterin, R5020, to more precisely measure the distribution of the hormone-binding competent sPR and tPR between the two fractions (Table I). For sPR, there was six times more hormone binding activity in the cytosolic fraction than the nuclear fraction. In contrast, hormone binding activity of tPR was approximately equal between the cytosolic and nuclear fractions. This difference in fractionation of sPR and tPR is statistically significant and supports the previous observation that the two functionally distinct forms of the receptor have differential nuclear binding properties when unliganded.

To determine whether PR in the nuclear and cytosolic fractions had differential hormone binding affinity, Scatchard analysis was performed on the sPR with cytosolic and nuclear extracts using R5020. There was no significant difference in affinity to R5020 between the two fractions. The Kd value for sPR in the cytosolic extract was 0.775 nM as compared with 0.815 nM in the nuclear extract. The Bmax values, which indicate the concentration of receptor, were 208 fmol/mg for the cytosolic extract, and 101 fmol/mg for the nuclear extract. Approximately equal amounts of protein were assayed. This is consistent with the one-point hormone binding data that revealed that more of the hormone-binding competent sPR was present in the cytosolic fraction. It was not feasible to perform this comparison with the tPR considering the amount of protein required for this assay. As previously reported, cellular expression of sPR and tPR was approximately equal in all experiments (data not shown).

Composition of PR-Chaperone Complexes in the Two Fractions—The presence of two distinct fractions of the unliganded PR raises two immediate questions. First, do the two fractions differ in terms of the composition of PR complexes? Second, is the composition of the complexes different between the two functionally distinct forms of PR? In the unliganded state, the PR has been shown to associate with chaperone proteins in an ordered sequence ultimately resulting in a mature, hormone-binding competent complex containing hsp90, p23, and a large immunophilin, preferentially FKBP51 (25). To examine the chaperone composition of PR complexes and determine whether the sPR and the tPR are processed differently, a series of immunoprecipitation experiments were performed. Because the results of the hormone binding data indicated differences in cellular distribution of the hormone-binding competent PR, the focus was on isolating, identifying, and comparing cellular distribution of the mature PR complex.

Hsp90 is an important member of the chaperone complex because it binds PR, p23, and large immunophils through non-overlapping sites. To examine PR-hsp90 interactions, we prepared cytosolic and nuclear extracts from mouse mammary adenocarcinoma cells, either untreated or treated with R5020. Extracts were generated in the presence of tunicamycin, which, like molybdate, stabilizes hsp90 interactions with steroid hormone receptors (26). However, unlike molybdate, tunicamycin retains this activity in high salt conditions (27).

Receptor was immunoprecipitated from extracts containing approximately equal amounts of sPR and tPR using the PR22 antibody, which recognizes both A and B forms of the receptor.

Immunoprecipitates were immunoblotted with hsp90 antibody to determine relative amounts of hsp90-PR complex formation with sPR and tPR in cytosolic and nuclear fractions, as shown in Fig. 1. In both nuclear and cytosolic extracts, unliganded PR associated with hsp90 (Fig. 1A, lanes 8 and 9 and 17 and 18) and this association was significantly decreased in the presence of hormone (Fig. 1A, lanes 6 and 7 and lanes 15 and 16). This is consistent with the original finding that hsp90 associates with PR in the absence of hormone (28, 29). By immunoprecipitating with extracts containing approximately equal amounts of PR and hsp90 (Fig. 1A, lanes 1–4 and 10–13), we determined that, in the absence of progesterin, there was significantly more hsp90/PR association in the cytosolic fraction as compared with the nuclear fraction (Fig. 1A, compare lanes 8 and 9 with 17 and 18). To provide a quantitative analysis, hsp90 levels in the immunoprecipitates were normalized to immunoprecipitated PR levels for each sample as shown (Fig. 1B). For both the tPR and sPR there was approximately one-third the amount of hsp90 association in the nuclear extracts relative to the cytosols. The amount of associated hsp90 in each extract was similar for unliganded sPR and tPR. Next, association with the co-chaperone p23 was examined. The p23 protein is an essential component of the mature PR complex, stabilizing the complex through its association with hsp90 (15, 30). Attempts to measure p23 association with immunoprecipitated PR were complicated by high levels of non-specific binding of p23 to the antibody resin. Therefore, we measured PR association with immunoprecipitated p23 in ex-
tractions containing equal amounts of sPR and tPR, as shown in Fig. 2. As shown for hsp90, PR association with p23 was ligand-dependent in all cases (Fig. 2, compare lanes 2, 4, 6, and 8 with lanes 3, 5, 7, and 9). In the unliganded state, receptor association with p23 was similar for sPR and tPR in both nuclear and cytosolic extracts.

Association of PR and FKBP51 was then examined. FKBP51 has been shown previously to be the preferred immunophilin in mature PR complexes (25, 31). We immunoprecipitated PR from the various extracts and examined association of FKBP51 by immunoblotting as shown in Fig. 3. Surprisingly, FKBP51 association with PR occurred predominantly in the nuclear fraction for both sPR and tPR (Fig. 3A, compare lanes 7 and 9 with 2 and 4), although there were significant amounts of FKBP51 protein in both nuclear and cytosolic extracts (Fig. 3B). Although it appears that there is a higher degree of FKBP51/receptor association in nuclear extracts containing sPR, this was not consistent in other experiments. As in the case of hsp90 and p23, hormone treatment significantly reduced association of FKBP51 with PR (Fig. 3A, compare lanes 2, 4, 7, and 9 with 3, 5, 8, and 10). The FKBP52/59 protein did not co-immunoprecipitate with PR under these conditions, although it is expressed in these cells (data not shown).

Dimerization of Unliganded PR Occurs Predominantly in the Tightly Bound Nuclear Fraction—The binding of ligand to steroid hormone receptors induces a transformation of the receptor that allows it to dimerize and bind DNA and coactivator proteins (32). The PR, having the A and B isoforms, can form PRA/PRB heterodimers as well as PRA or PRB homodimers (33). The reduced association of PR with hsp90 in the nuclear extracts raised the possibility that the PR might be partially transformed and dimerized in the absence of progestin. To test this hypothesis, we carried out immunoprecipitation with an antibody recognizing only the B isoform of PR, followed by Western blotting with the PR22 antibody, which recognizes both A and B isoforms. The presence of the A isoform in the immunoprecipitates indicates the formation of PRA/PRB heterodimers. The results are shown in Fig. 4. The immunoprecipitates blotted with PR22 (Fig. 4A, lanes 3–12) showed a slower migrating species corresponding to PRB and a series of faster migrating species, which were slightly upshifted from the migration of PRA in the input nuclear extracts (compare lanes 2 and 3 with 6 and 7). Phosphorylation of PRA at a serine residue in the amino-terminal domain has been reported to slow its migration on SDS-PAGE (34). To ensure that these upshifted bands correspond to phosphorylated forms of PRA.

Comparing the immunoprecipitated samples, we observed significantly more PRA/PRB heterodimers in the nuclear frac-
lanes 6–10) extracts containing either sPR or tPR, as indicated. Cells treated with 30 nM R5020 are indicated (+). Extracts incubated with Protein A-Sepharose beads alone, designated as B, were included as controls. B, immunoblot of FRBP51 and PR from cytosolic and nuclear extracts.

Fig. 3. FKBP51 association with PR in the two biochemical fractions. A, an immunoblot of FKBP51 and PR after immunoprecipitation (IP) with PR22 in cytosolic (lanes 1–5) and nuclear (lanes 6–10) extracts containing either sPR or tPR, as indicated. Cells treated with 30 nM R5020 are indicated (+). Extracts incubated with Protein A-Sepharose beads alone, designated as B, were included as controls. B, immunoblot of FRBP51 and PR from cytosolic and nuclear extracts.

tions than in the cytosolic fractions (Fig. 4A, compare PRA intensity in lanes 3–6 with that of lanes 8–11), indicating that the nuclear fraction contains greater levels of dimerized, chaperone-free PR. This is consistent with the results of Fig. 1, which show that less hsp90 immunoprecipitates with PR in the nuclear extracts. Comparing nuclear fractions, the level of heterodimerization for the unliganded tPR was on average, three times higher than for unliganded sPR when the average relative ratios of PRA to PRB were calculated in the immunoprecipitates (Fig. 4B). In the input extracts the average PRA/PRB ratio appears to be slightly higher for tPR relative to sPR, but the difference was not statistically significant. Although the data in Fig. 4A suggests that less heterodimerization was observed after hormone treatment, there was no statistically significant difference in levels of heterodimers formed in untreated cells compared with those treated with R5020 based on results from four independent experiments (data not shown).

sPR and tPR Respond Similarly to Geldanamycin—Our results indicate that a large pool of cellular tPR may exist free of chaperones, raising the possibility that a fraction of the tPR may not be processed through the chaperone cycle. To test this hypothesis, cells were treated with the benzoquinone ansamycin, geldanamycin. This drug binds to hsp90 at its amino-terminal ATP-binding site, and inhibits its ATPase activity (35). Geldanamycin has previously been shown to inhibit mature complex formation and PR and glucocorticoid receptor (GR) function by preventing association of p23 to hsp90. This results in a loss of ligand-induced transcriptional activity (30, 36, 37). If a significant fraction of tPR escapes the chaperone cycle, it may be partially resistant to the effects of geldanamycin on its ability to activate transcription. Cells expressing either sPR or tPR were treated with varying amounts of geldanamycin 30 min prior to addition of R5020. The ability of sPR and tPR to activate a transfected MMTV-luciferase reporter was measured (Fig. 5). In both cases geldanamycin inhibited PR-dependent transactivation with similar EC₅₀ values, ~6.25 and 6.53 ng/ml for sPR and tPR, respectively. This finding reveals that both sPR and tPR are processed through the chaperone cycle equally and form the mature, hormone-binding competent complex.

The Majority of the Nuclear PR Associates with Proteins That Are Not Components of the Nuclear Matrix—The requirement of high ionic strength conditions for PR nuclear extraction implies that PR may be binding to nuclear components. Estrogen receptors, which reside mainly in the nucleus, have been shown to associate with the nuclear matrix (21, 38). We further extracted nuclei from cells expressing tPR or sPR to determine whether PR is bound to DNA, protein, and/or the nuclear matrix. After isolation of nuclei from 30T1.7 cells or 1470.2 cells expressing tPR, they were extracted with various combinations of salt, DNase, and Triton X-100. After extraction, nuclei were pelleted and supernatants were collected. Equal fractions of each sample were blotted with the PR antibody. Fig. 6A shows that most of the full-length receptor in the nucleus was eluted with salt and that only a very small amount of full-length sPR and tPR remained associated with the nucleus. DNase treatment without salt extraction did not result in the release of PR from the nucleus (Fig. 6A, lanes 2 and 7), indicating that the receptor is not retained in the nucleus because of DNA binding alone. Further extraction of salt- and DNase-treated nuclei with Triton X-100 did not result in any further release of PR (Fig. 6A, lanes 4 and 9). The nuclear pellet after extraction with salt, DNase, and Triton X-100 contained a very small amount of full-length PR, indicating that most of the tightly associated nuclear PR does not associate with the nuclear matrix (Fig. 6A, lanes 5 and 10).

There was a significant amount of a truncated (~60,000 molecular weight) form of the tPR, which was found in the nuclear pellet (Fig. 6A, lane 5). To determine which portion of the receptor was represented by this truncated form, an immunoblot was performed with this fraction using the PR6 antibody, which recognizes the amino-terminal region of PR only present in the B isoform (Fig. 6B). The results show that the smaller tPR form contains the amino-terminal domain, indicating that the truncation occurs in the carboxyl-terminal domain. Based on this result and the size of the fragment, it is unlikely that this truncated form contains a functional hormone-binding domain and responds to ligand in our system. Its tight association with the insoluble fraction of nuclei suggests that it may not be able to interfere with receptor activities that require mobility, such as transcription and chaperone processing. The truncated tPR was not observed in the cytosolic or nuclear fractions in previous experiments, as it was not eluted in high ionic strength buffers.

tPR Cannot Bind to a Stably Replicating MMTV Promoter—We showed previously that the tPR cannot activate the MMTV promoter in organized, replicating chromatin, and it fails to induce the remodeling of chromatin necessary for transactivation. Given that a significant fraction of the unliganded tPR has altered nuclear binding properties, reduced association with chaperones, and is partially transformed, it is possible that this status diminishes its ability to interact with binding sites in chromatin or recruit the proper remodeling machinery. To determine whether the failure of the tPR to activate transcription from a stably replicating promoter was because of its inability to bind to the promoter, we carried out DNA FISH and indirect immunofluorescence in 3134 cells. These cells contain a 200-repeat tandem MMTV array that can be easily visualized by DNA FISH and, as in 1470.2 cells, the tPR is unable to activate the MMTV promoter in this array (13). Stable expression of a green fluorescent protein-tagged GR in these cells has also allowed visualization of the MMTV array in the presence of glucocorticoids because of GR accumulation at the hormone responsive elements (19).

In combination with DNA FISH, indirect immunofluorescence was used to visualize the association of various factors with the MMTV array. In Fig. 7 (panels A–F), we measured PR localization in the presence of ligand in combination with DNA
FISH using two PR antibodies that recognize completely different epitopes. We observed several punctate regions of localization per nucleus. However, the vast majority of cells showed no measurable colocalization with either antibody; colocalization of the PR with the MMTV array was observed in only a small number of cells visualized by DNA FISH. CBP has been shown in numerous studies to act as a nuclear receptor cofactor. Specifically it has been shown that CBP has a synergistic effect in combination with R5020 on the transcriptional activity of the chicken PR (39). In addition, CBP has been shown previously to associate with the MMTV array upon activation of GR and the induction of transcription at the MMTV promoter (24). As a positive control, immunostaining with CBP was performed in the presence of dexamethasone. Under these conditions, ligand-dependent co-localization of CBP and the stably replicating MMTV promoter was visualized in more than 60% of the cells analyzed (Fig. 7, panels G–I). As a negative control, cells were treated with R5020 followed by MMTV-FISH and CBP staining. Under these conditions, co-localization of CBP and MMTV was visualized in a relatively small number of cells, similar to that observed with PR (Fig. 7, panels J–L) and consistent with a general lack of PR association with the MMTV promoter in organized chromatin.

**DISCUSSION**

Our previous studies detailed profound functional differences between two forms of the PR in mammary adenocarcinoma cells, one transiently expressed and one constitutively expressed. These functionally distinct PRs differed in their ability to activate a target gene requiring chromatin remodeling, their sensitivity to progestins, and their ability to be activated by other signaling pathways in a ligand-independent fashion. In this study, we have characterized the two forms of PR in terms of their cellular distribution and nuclear binding properties, their interactions with chaperone proteins, and

### FIG. 4. PR dimerization in the two biochemical fractions. A, lanes 1 and 2 represent an immunoblot of non-immunoprecipitated nuclear (N) and cytosolic (C) extracts containing sPR. Lanes 3–12 show an immunoblot with the PR22 antibody (recognizing both PRA and PRB isoforms) after immunoprecipitation with the PR6 antibody (recognizing only PRB) in nuclear (lanes 3–7) and cytosolic (lanes 8–12) extracts containing either sPR or tPR, as indicated. Non-transfected cell extracts (Nt) were included as controls. PR6-immunoprecipitated nuclear extracts blotted with the PR6 antibody are shown in lanes 13 and 14. Extracts from cells treated with 30 nM R5020 are indicated (+). B, graphical representation of the relative levels of sPR and tPR heterodimerization in the nuclear fraction. Data derived from non-immunoprecipitated extracts (No IP) and immunoprecipitated extracts (IP) from untreated cells expressing either sPR or tPR are shown. The data on the graph represent the average and standard deviation of three independent experiments (No IP) and four independent experiments (IP). For each of the independent experiments the ratio of PRA signal intensity to PRB signal intensity was calculated for both sPR (s) and tPR (t). In each case the ratio for sPR was set to 1 and the ratio for tPR was expressed as a multiple. Statistical significance (Student’s t test; p < 0.05) is indicated with an asterisk.

### FIG. 5. Geldanamycin (GA) dose response curves for tPR (A) and sPR (B). Cells were transfected with pLTRluc and pcPRO (1470.2 cells only) and treated with various concentrations of geldanamycin 30 min prior to the addition of R5020. After 4 h of subsequent treatment, cells were harvested. Cell extracts were subjected to assays for protein and luciferase. Fold induction was calculated relative to untreated cells. Dose response curves and EC50 values were generated using GraphPad Prism software.

### FIG. 6. PR associates with proteins other than nuclear matrix components. A, nuclei were extracted as indicated under “Experimental Procedures.” Proteins in the resulting supernatants (supe) or the pellet (P) were electrophoresed on a 8% polyacrylamide gel and immunoblotted with the PR22 antibody. The data shown are from one representative set of three independent experiments. B, immunoblot of the pellet (P) fraction from cells expressing tPR using the PR6 antibody, which recognizes an epitope in the amino-terminal domain unique to PRB.
 their dimerization status. These results provide new information about the diversity and cellular localization of specific chaperone-receptor complexes and the nature of the unliganded receptor in mammary adenocarcinoma cells. These data also suggest that the equilibrium between these complexes can have a profound effect on PR function and indicate that the often used transiently expressed steroid receptor may not be a complete model for steroid receptor function.

Unliganded steroid receptors were initially thought to reside in the cytoplasm complexed with chaperones. Upon binding to their specific ligand, the receptors were thought to translocate to the nucleus where they associated with chromatin. This was based on biochemical fractionation experiments similar to those carried out in this study in which the requirement for extraction under conditions of high ionic strength was used to differentiate between nuclear and cytosolic extracts. The development of antibodies against steroid receptors led to indirect immunofluorescence experiments showing that some steroid receptors, such as PR and ER, were mostly localized to the nucleus in the absence of ligand (40, 41), even though biochemical methods indicated that these receptors were cytoplasmic (42). This behavior implies that unliganded receptors are not tightly bound to the nucleus. In accordance, studies have shown that unliganded receptors, when associated with chaperones, cannot bind DNA or co-factors and shuttle constantly between the cytoplasm and the nucleus (43–45). Many of the original biochemical fractionation studies were carried out with cells or tissues that endogenously express the receptor of interest. In our study the fractionation behavior of the constitutively expressed sPR resembles that of these endogenous receptors with the vast majority of the unliganded but hormone-binding competent receptor fractionating with the cytosol. In contrast, the tPR has a higher affinity for the nucleus in the absence of ligand, with almost half requiring salt extraction. A small amount of the unliganded sPR (15%) also requires salt extraction. To our knowledge, the existence and characterization of such unliganded but tightly bound nuclear PR complexes has not previously been documented.

Through hormone binding assays and receptor co-immunoprecipitation experiments, we found that hormone binding and chaperone association occurred in both nuclear and cytoplasmic fractions. However, distinct receptor/chaperone interactions occurred in each fraction. PR associated with hsp90 and p23 in both fractions. Whereas the level of PR association with p23 appeared to be similar, hsp90 association was reduced in the nuclear fraction. Because p23 associates with receptors through binding to hsp90, this was surprising. However, it has been suggested that p23 may interact with nuclear receptors bound to DNA and facilitate their release in the absence of hsp90 by an unknown mechanism (46). In addition, the experiments to detect PR association with hsp90 and p23 were performed differently for technical reasons. In the case of hsp90, the immunoprecipitating antibody bound PR and presumably brought down all PR complexes, including those without chaperones. In the case of p23, the immunoprecipitating antibody bound to p23 and brought down only those complexes in which PR was associated with p23. Our results show that some of the nuclear PR is dimerized and presumably free of chaperones (discussed below).

In agreement with other studies was the finding that FKBP51 preferentially associates with chaperone complexes containing PR (25), because we did not detect FKBP52 association with PR. However, one major difference between PR-chaperone interactions in the two fractions was the unique presence of FKBP51 in the PR complexes immunoprecipitated from the nuclear extracts. Although FKBP51 has been found in PR complexes immunoprecipitated from chicken oviduct cytosol and reticulocyte lysates, we did not detect FKBP51 association with PR in the cytosolic fraction. It is possible that the FKBP51-containing complex in this fraction is less stable under the experimental conditions in our study or that a different protein containing tetratricopeptide repeat domains is associated with the PR-chaperone complex in this fraction.

FKBP51 function with respect to PR is not fully understood. In squirrel monkeys, FKBP51 has been linked to cortisol resistance (47). In yeast, FKBP52 increases the affinity of GR for its ligand (48). Effects of FKBP51 on PR activity appear to be variable. FKBP51 association with PR did not have an effect on

![Diagram](https://example.com/diagram.png)

**Fig. 7.** tPR associates with the stably replicating MMTV promoter in a small percentage of cells. Panels A–F: MMTV DNA FISH and indirect immunofluorescence of PR in R5020-treated cells. PR immunofluorescence was carried out with the PR6 antibody (panels A–C) and the PR22 antibody (panels D–F). Panels G–L: MMTV DNA FISH and indirect immunofluorescence of CBP. Cells in panels G–I were treated with dexamethasone (100 nM). Cells in panels A–F and J–L were treated with R5020 (30 nM). Antibody staining is represented in panels A, D, G, and J. MMTV-FISH staining is represented in panels B, E, H, and K. The overlay of antibody and MMTV-FISH staining is represented in panels C, F, I, and L. A 0.5-μm scale bar is shown in the overlay panels. The percentage of cells that displayed co-localization of the array and the antibody signals for each condition is shown on the right (n = 24–44).
its affinity for hormone in our system. However, a recent study showed that in HepG2 cells, overexpression of FKBP51 reduced progestin responsiveness (49). Freeman and Yamamoto (50) have provided compelling evidence that hsp90 and its co-chaperone p23 are involved in the removal of hormone-exposed or “experienced” steroid receptors from chromatin, thus attenuating their transcriptional activity and preparing the receptor for recycling. Hache et al. (51) have shown that GR is retained in the nucleus after hormone withdrawal even though it is reassociated with chaperones. The unique association of FKBP51 with unliganded nuclear PR-chaperone complexes leads us to speculate that it may be part of the chaperone complex involved in attenuation of steroid receptor signaling.

Another unique feature of the unliganded PR fractionating with nuclear extracts is the existence of PR heterodimers. According to the classic model of steroid receptor cycling, unliganded receptors are held in chaperone complexes as monomers. Upon binding to ligand, the receptors become free from chaperone interaction and dimerize as part of the transformation process. The presence of PR heterodimers in the absence of ligand indicates either that this pool of PR did not participate in the chaperone cycle or that it became at least partially transformed by a ligand-independent mechanism. We favor the latter explanation because there is an overwhelming amount of evidence that the chaperone cycle is critical for proper folding and functionality of steroid receptors. In addition, both sPR and tPR were inhibited to the same extent by geldanamycin, which binds hsp90 and blocks formation of the hormone-binding competent receptor complex. This observation implies that although approximately half of the tPR has an altered nuclear distribution, it is dynamic and still participates in the chaperone cycle. In addition, we observed that the level of unliganded tPR heterodimers in the nuclear extract was approximately three times higher than that of unliganded sPR heterodimers, perhaps indicating that the nuclear fraction of the tPR contains an overall higher fraction of heterodimerized, partially transformed PR.

From these observations, the model in Fig. 8 was generated. It shows the two receptors in the unliganded state with the percentage of hormone-binding competent receptor in each fraction indicated. Association with hsp90, p23, and FKBP51 in each fraction are represented in each fraction. We suggest that the cytoplasmic or loosely bound nuclear fraction of PR can be converted by ligand-independent means to a chaperone-free, dimerized state that is tightly bound to the nucleus. Based on our data, this may occur with greater frequency for the tPR than the sPR. A remaining question is the nature of the trigger for this altered equilibrium and cellular distribution. The answer may lie in the multitude of signal transduction pathways involved in post-translational processing events such as phosphorylation, because the dimerized PR appears to have a higher level of phosphorylation (Fig. 4A). We also propose that the tightly bound nuclear complex containing PR, hsp90, p23, and FKBP51 may serve to transition the PR from the dimerized state back into the chaperone cycle. Further study of the role of FKBP51 in our system is required to resolve this issue.

The model in Fig. 8 also suggests the possibility that the dimerized, chaperone-free form of the PR may bind promoters with accessible chromatin structure even in the absence of ligand. DNase I treatment of nuclei isolated under low ionic strength conditions did not result in the release of tightly bound nuclear PR but we cannot rule out the possibility of DNA binding because the PR may associate with promoters through a combination of DNA and protein interactions. However, our imaging experiments show that the tPR, which is much more enriched in the tightly bound nuclear fraction, does not stably associate with a target promoter requiring chromatin remodeling for activation in the majority of cells in which it is expressed. Just over half of the hormone-binding competent tPR appears to be in the loosely bound cytoplasmic state and thus

![Figure 8: Model of unliganded sPR and tPR cycling in murine mammary adenocarcinoma cells.](http://www.jbc.org/Downloadedfrom)
Partially Transformed PR Cannot Bind Chromatin

10. Smith, C. L., Archer, T. K., Hamlin-Green, G., and Hager, G. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11202–11206
11. Smith, C. L., Woldfard, R. G., O’Neill, T. B., and Hager, G. L. (2000) Mol. Endocrinol. 14, 956–971
12. Richard-Foy, H., and Hager, G. L. (1987) EMBO J. 6, 2321–2328
13. Smith, C. L., Hun, H., Woldfard, R. G., and Hager, G. L. (1997) J. Biol. Chem. 272, 14227–14235
14. Smith, D. F. (2000) Semin. Cell Dev. Biol. 11, 45–52
15. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) Mol. Cell. Biol. 15, 6804–6812
16. Kosano, H., Stengard, B., Charlesworth, M. C., McMahon, N., and Toft, D. (1998) J. Biol. Chem. 273, 32973–32979
17. Pennine, W. D., Hager, G. L., and Smith, C. L. (1995) Mol. Cell. Biol. 15, 2125–2134
18. Groumewer, H., Turetto, B., Quinrin-Stricker, C., Bouquet, M. T., Meyer, M. E., Krouzewski, Z., Jeltsch, J. M., Lerouge, T., Garnier, J. M., and Chambon, P. (1987) EMBO J. 6, 3885–3994
19. McNally, J. G., Muller, W. G., Walker, D., Woldfard, R. G., and Hager, G. L. (2000) Science 287, 1282–1285
20. Giordano, T., Howard, T. H., Coleman, J., Sakamoto, K., and Howard, B. H. (1991) Exp. Cell Res. 192, 193–197
21. Sun, J.-M., Chen, H. Y., and Davie, J. R. (2001) J. Biol. Chem. 276, 49433–49442
22. Johnson, J., Corbissier, R., Stengard, B., and Toft, D. (1996) J. Steroid Biochem. Mol. Biol. 56, 31–37
23. Logeat, F., Pamphile, R., Lassaf, H., Jolivet, A., Fournier, A., and Milgrom, E. (1985) Biochemistry 24, 1029–1035
24. Muller, W. G., Walker, D., Hager, G. L., and McNally, J. G. (2001) J. Cell Biol. 154, 53–58
25. Nair, S. C., Rimerman, R. A., Toran, E. J., Chen, S., Prapapanich, V., Butt, R. N., and Smith, D. F. (1997) Mol. Cell. Biol. 17, 594–603
26. Hartson, S. D., Thulasiraman, V., Huang, W., Whitesell, L., and Matts, R. L. (1999) Biochemistry 38, 3837–3846
27. Renoir, J. M., Radany, C., Jung-Testas, I., Faber, L. E., and Baulieu, E. E. (1990) J. Biol. Chem. 265, 14402–14406
28. Sanchez, E. R., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1985) J. Biol. Chem. 260, 12398–12401
29. Castelli, M. G., Binart, N., Jung-Testas, I., Renoir, J. M., Baulieu, E. E., Firmoso, J. R., and Welch, W. J. (1985) EMBO J. 4, 3131–3135
30. Johnson, J. L., and Toft, D. O. (1995) Mol. Endocrinol. 9, 670–678
31. Barent, R. L., Nair, S. C., Carr, D. C., Ruan, Y., Rimerman, R. A., Fulton, J., and Yang, Z., and Smith, D. F. (1998) Mol. Endocrinol. 12, 342–354
32. Truss, M., and Beato, M. (1995) Endocr. Rev. 16, 469–479
33. Leonhardt, S. A., Alltmann, M., and Edwards, D. P. (1998) Mol. Endocrinol. 12, 1914–1930
34. Bai, W., and Weinigel, N. L. (1996) J. Biol. Chem. 271, 12801–12806
35. Soti, C., Racz, A., and Ceerency, P. (2000) J. Biol. Chem. 277, 7066–7075
36. Whitesell, L., and Cook, P. (1996) Mol. Endocrinol. 10, 705–712
37. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Cell 89, 239–250
38. Stenoien, D. L., Mancini, M. G., Patel, K., Allegretto, E. A., Smith, C. L., and Mancini, M. A. (2000) Mol. Endocrinol. 14, 518–534
39. Rowan, B. G., Garrison, N., Weinigel, N. L., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 8720–8730
40. King, W. J., and Greene, G. L. (1984) Nature 307, 745–749
41. Perrot-Applanat, M., Logeat, F., Groyer-Picard, T. M., and Milgrom, E. (1985) Endocrinology 116, 1473–1484
42. Gorski, J., Toft, D., Shymalal, G., Smith, D., and Ntideis, A. (1968) Recent Prog. Horm. Res. 24, 45–80
43. Gisouin-Mantel, A., Delabre, K., Lescop, P., and Milgrom, E. (1996) J. Steroid Biochem. Mol. Biol. 56, 3–9
44. Yang, J. L., and DeFranco, D. B. (1997) J. Cell Biol. 137, 523–538
45. Pratt, W. B. (1995) J. Biol. Chem. 270, 21455–21458
46. Freeman, B. C., Felts, S. J., Toft, D. O., and Yamamoto, K. R. (2000) Genes Dev. 14, 422–434
47. Reynolds, P. D., Ruan, Y., Smith, D. F., and Scammell, J. G. (1999) J. Clin. Endocrinol. Metab. 84, 663–669
48. Riggs, D. L., Roberts, P. J., Chrillo, S. C., Cheung-Flynn, J., Prapapanich, V., Ratajczak, T., Gaber, R., Picard, D., and Smith, D. F. (2003) EMBO J. 22, 1–10
49. Hubscher, T. R., Denny, W. B., Valentine, D. L., Cheung-Flynn, J., Smith, D. F., and Scammell, J. G. (2003) Endocrinology 144, 2380–2387
50. Freeman, B. C., and Yamamoto, K. R. (2002) Science 296, 2232–2235
51. Hache, R. J. G., Tse, R., Reich, T., and Savory, G. A. (1999) J. Biol. Chem. 274, 1432–1439
Progesterone Receptor Deficient in Chromatin Binding Has an Altered Cellular State
Jeannine Botos, Wenjuan Xian, David F. Smith and Catharine L. Smith

J. Biol. Chem. 2004, 279:15231-15239.
doi: 10.1074/jbc.M309718200 originally published online January 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309718200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 25 of which can be accessed free at http://www.jbc.org/content/279/15/15231.full.html#ref-list-1