Stoichiometry and Site-specific Phosphorylation of Human Progesterone Receptor in Native Target Cells and in the Baculovirus Expression System*

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Human progesterone receptor (PR) in T47D breast cancer cells is phosphorylated on nine different serine residues; three are hormone-inducible (Ser102, Ser294, and Ser345), while others are basal but hormone-stimulated. In the present study, we have compared the phosphorylation state of native and recombinant PR expressed in a baculovirus insect cell system. Stoichiometric measurements showed that unliganded native PR in T47D cells was approximately 50% phosphorylated (∼4 phosphates/PR) and became essentially 100% phosphorylated (∼9 phosphates/PR) when bound to hormone. Unliganded PR expressed in Sf9 insect cells was phosphorylated with a similar stoichiometry (∼3 phosphates/PR), but the phosphate content did not change with hormone addition. Site-specific phosphorylation analyzed by tryptic phosphopeptide mapping and manual peptide sequencing revealed that expressed PR bound to hormone in the Sf9 insect cells was phosphorylated on the same sites as hormone-treated PR in T47D cells. Only minor differences were detected in the relative proportion of three sites (two basal sites and Ser345) and phosphorylation did not occur on alternate sites. Interestingly, unliganded baculovirus-expressed PR was constitutively phosphorylated on hormone-inducible sites and was phosphorylated on basal sites to the same extent as hormone treated PR. Thus, in the absence of hormone, the phosphorylation state of baculovirus-expressed PR resembled that of the hyperphosphorylated native PR. In contrast to native PR, the expressed receptor in cytosols of Sf9 cells did not form a large oligomeric complex suggesting that hyperphosphorylation may be due to dissociation of the complex in the absence of hormone. This study demonstrating phosphorylation on correct sites with a stoichiometry similar to that of native PR indicates that overexpressed PR in the baculovirus system is suitable for in vitro structure/function studies.

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§ The abbreviations used are: PR, progesterone receptor; ER, estrogen receptor; GR, glucocorticoid receptor; hsp90, heat shock protein 90; hsp70, heat shock protein 70; HPLC, high pressure liquid chromatography; R5020, pronegestone; RU486, Mifapristone; ZK98299, Onaprisone; TPCK, tosylphenylalanyl chloromethyl ketone.
and PR (27, 28) have all been reported to be expressed at high levels in insect cells (up to 3–5% of total soluble protein) and to exhibit functional activities for steroid binding. DNA binding, and transcription that are similar to that of native receptors (19, 21, 23, 25, 27–30).

Little work has been done to investigate the manner in which recombinant steroid receptors expressed in insect cells are post-translationally modified by phosphorylation. The glucocorticoid receptor (20), estrogen receptor (31), and our earlier studies with human PR (27) have shown by immunoprecipitation of \( ^{32} \)P-labeled protein that receptors do become phosphorylated when expressed in insect cells. Only one study with ER has performed phosphopeptide mapping to characterize the sites of phosphorylation (31). Because of the widespread use of the baculovirus system for overexpression of steroid receptors and its utility as a source of receptor for structural studies, it is important to know whether expressed receptors are correctly phosphorylated. In the present study we evaluated by phosphopeptide mapping and manual amino acid sequencing whether human PR expressed in the baculovirus system is phosphorylated correctly on the same sites as the native protein. We have also determined the stoichiometry of phosphorylation and how hormone affects overall and site-specific phosphorylation of the recombinant receptor.

**EXPERIMENTAL PROCEDURES**

Materials—\( ^{3} \)HJRSO20 (promegestone: \( 17a \text{-methyl-}^{3} \text{H}) \text{I}7a,21 \text{-di}-
19\text{-norpregn-4-}a,3\text{-20-one: 87 Ci/mmol}, \text{unlabeled R5020, carrier-free} \( ^{3} \)P\text{H}_{3}\text{PO}_{4} (8200–9200 Ci/mmol), and EXPRESS-35S protein labeling mix (1.175 Ci/mmol) were obtained from DuPont NEN. The progesterin antagonist, RU486 (17-hydroxy-12,14-dimethyl-
17\text{-pregnen-3-}a\text{-ol}) was a gift from Roussel-UCLAF (Romainville, France), and ZK92899 was provided by David Henderson (Schering, Berlin). AB52 is a mouse monoclonal antibody produced against purified human PR that recognizes both the B and A isoforms of PR (32). Tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington. Gel electrophoresis supplies were from Bio-Rad.

Cell Culture, Radiolabeling, and Receptor Preparation—T47D hu-
man breast cancer cells were cultured and labeled to steady-state with \( ^{3} \)P as described previously (33), and recombinant PR was produced from baculovirus vectors in Sf9 insect cells as also described previously (27). To label PR expressed in Sf9 insect cells, 20 \( \times \) 106 cells (after 42 h of incubation) were incubated for 1 h at room temperature in phosphate-free EX-CELL401 medium (J RH Biosciences, Lenexa, KS). Cells were then collected by centrifugation and resuspended in 5 ml of phosphate-free EX-CELL401 containing 0.833 mM [\( ^{3} \)P]orthophosphate and incubated for another 4 h at room temperature followed by addition of vehicle (ethanol) or hormone for an additional 2 h. Harvested cells were washed in homogenization buffer (KPFM: 50 mM potassium phosphate, pH 7.4, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol) and lysed in KPFM containing 0.5 mM NaCl and a mixture of protease inhibitors (16). Cell lysates were centrifuged at 100,000 \( \times \) g for 30 min, and the supernatant, considered as a whole cell extract, was dialyzed against KPFM or diluted 1:2 with KPFM to reduce salt concentration before immunoprecipitation. For labeling with \( ^{35} \)S, alternative cultures of infected Sf9 cells were labeled above with methionine-free EX-CELL401 for 1 h before incubation with methionine-free EXCELL401 containing 25 \( \mu \)Ci/ml [\( ^{35} \)S]methionine for a total of 6 h. Hormone or vehicle were added during the last 2 h of labeling.

Immunoprecipitation and Gel Purification of PR—Radiolabeled PR in dialyzed or diluted whole cell extracts were immunoprecipitated with the human PR specific monoclonal antibody AB-52 using protein A-Sepharose as described previously (16, 33, 34). PR was eluted from protein A-Sepharose with 2% SDS-sample buffer, electrophoresed on 7.0% discontinuous SDS-polyacrylamide gels as described previously and the gel pieces containing PR bands (detected by autoradiography of wet gels) were excised and the incorporated radioactivity was quanti-
tated by Cerenkov counting.

Trypsin Digestion and Phosphopeptide Analysis—Radiolabeled PR in excised gel pieces were eluted and digested with TPCK-treated trypsin as described previously (33). The [\( ^{32} \)P]labeled tryptic peptides were separated on a Vydyac C18 reverse phase column controlled by a Gilson HPLC system using a 0–45% \( \text{H}_{2}\text{O}: \text{acetonitrile gradient containing 0.1% trifluoroacetic acid. The column was run for 90 min at a flow rate of 1 ml/min as described previously (14, 15, 33). \( ^{32} \)P]-labeled peptides were detected by on-line p-AM radio-HPLC detection from Microwave Systems (Tampa, FL) using a 0.5-ml flow cell. In some experiments tryptic phosphopeptides were also separated by electrophoresis on an 40% alkaline polyacrylamide gel as described previously (14). The gel was dried, and phosphopeptides were detected by autoradiography.

**Release of 32P from PR**—To determine the number and position of phosphorylated residues in each of the HPLC-separated phosphopeptides, we performed phosphopeptide radiolysis by the method of Sullivan and Wong (35). Briefly, each of the major phosphopeptide peaks collected by HPLC were dried down, dissolved in 30 ml of 50% acetonitrile, and covalently linked to an alylene-sequen-
disc as described previously (14, 15, 33). The disc was then subjected to Edman degradation by treatment for 3.0 min at 50 °C with 0.5 ml of coupling reagent (methanethiosulfonic acid-n-butylthioisocyanate; 7:1:1:1, v/v), followed by five washes with 1 ml of methanol, and re-
heated at 50 °C for 6 min with 0.5 ml of trifluoroacetic acid to cleave the alylene-amino terminal residue. The trifluoroacetic acid cleavage solution combined with a 1 ml of trifluoroacetic acid, 42.5% phosphoric acid (9:1) wash of the disc were quantitated for \( ^{32} \)P by Cerenkov counting. The disc was washed five times with 1 ml of methanol before the next cycle started.

**Stoichiometry of Phosphorylation**—To determine the moles of phos-
phate incorporated per receptor molecule, the specific radioactivity of the cellular ATP pools in Sf9 and T47D cells was measured under the \( ^{32} \)P labeling conditions used in this study. \( ^{32} \)P-ATP in cell lysates was extracted by precipitation with cold 12% trichloroacetic acid which was used to precipitate ATP in the supernatant, and ATP was for 

The cellular ATP pools in Sf9 and T47D cells was measured under the \( ^{32} \)P labeling conditions used in this study. \( ^{32} \)P-ATP was visualized by autoradiography and quanti-
tated by Cerenkov counting. Total cellular ATP concentrations were measured by an ATP determination kit according to the manufacturer's instructions (Sigma). In Sf9 cells the specific radioactivity of ATP was 652 \( \pm \) 17 (n = 3) dpm/pmol. In T47D cells the specific radioactivity of \( ^{32} \)P-ATP was 1,053 \( \pm \) 365 (n = 4) dpm/pmol. These values were used to convert disintegrations/min of \( ^{32} \)P incorporated in receptor into picomoles of phosphate. To determine the phosphate content per receptor mole, the picomoles of receptor in extracts submitted to immuno-
precipitation were determined by a single-saturating dose steroid bind-
ing assay using dextran-coated charcoal as the method for separation of free and receptor-bound ligand (37). The ligand was the synthetic pro-

gestin \( ^{3} \)HJRSO20. Because antibody was used in excess over PR under conditions where we can achieve a near quantitative immunoprecipita-
tion of receptor (37), we have made the assumption that all receptors in aliquots of cell extracts were immunoprecipitated and gel-purified. Even in the less than 100% method, this will provide a slight underestimate of the actual stoichiometry. More importantly PR in insect and T47D cell extracts were treated identically and should be immunoprecipitated with the same efficiency. Thus this method should provide an accurate determination of the phosphate content of recombinant PR relative to that of native PR.

In order for steroid binding to give an accurate phosphorylation of bacu-

virus-produced PR, the majority of the recombinant protein must be capable of binding steroid. In a previous study, we determined that \( \geq 80\% \) of baculovirus-expressed PR protein was capable of binding hor-

mone (38). This was confirmed in the present study by comparison of PR levels by steroid binding and a quantitative Western blot assay using PR in T47D cells as a standard (data not shown).

Approximately 10% of the cells were determined to be the phosphate content of native PR, we have also uniformly labeled total cellular protein in T47D cells by incubation with \( ^{35} \)S-methionine for 48 h as described previ-
ously (39) and have estimated the average specific radioactivity of total cellular pools of proteins. An aliquot of total \( ^{35} \)S-labeled proteins was precipitated from cell extracts by 25% trichloroacetic acid and the precipitate was quantitated by liquid scintillation counting. The total protein concentration was measured by Bradford assay and the average specific radioactivity of intracellular \( ^{35} \)S-labeled protein was calculated to be 4,819 \( \pm \) 961 (n = 3) dpm/pmol of protein. This value was also used with T47D cells to determine the picomoles of immunoprecipitated PR.

**Sucrose Density Gradient Centrifugation of PR**—Cytosols of Sf9 in-
sect cells or T47D cells were prepared in low ionic strength TED buffer (10 mM Tris-base, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) and were divided into two equal portions. One part was sup-
plemented with 50 mM sodium molybdate and the other had no further additions. Cytosols were then incubated for 3 h at 4 °C with \( ^{3} \)HJRSO20. At the end of the 3-h incubation, cytosols without molybdate were
supplemented with 0.4 M NaCl, and all samples were incubated for another 1 h at 4 °C for a total of 4-h exposure to hormone. Free steroid was removed with dextran-coated charcoal as described previously (40), and 200-μl aliquots of cytosols were layered on preformed 10–30% linear sucrose-density gradients. The gradients were prepared in TEDG buffer containing 50 mM sodium molybdate and centrifuged at 48,000 rpm for 16 h in a Beckman SW 55Ti swinging bucket rotor at 4 °C. Fractions of 100 μl were collected by a puncture hole through the bottom of the centrifuge tube and [3H]R5020 in each fraction was measured by liquid scintillation counting. As internal sedimentation markers, 14C-labeled bovine serum albumin (4.6 S) and 14C-labeled IgG (7 S) were centrifuged in the same gradients.

RESULTS
Recombinant PR Overexpressed in a Baculovirus System Is Phosphorylated on the Same Sites as Native PR—In previous studies we developed methods for tryptic phosphopeptide mapping in combination with secondary digestion and manual peptide sequencing to identify sites of phosphorylation of the native human PR in T47D breast cancer cells (14, 15). In the present study we have used these methods to determine whether baculovirus produced PR is phosphorylated on the same or different sites as native receptor. We have confined our analysis to the expressed full-length B form of PR which contains all the same major phosphorylation sites as PR-A, plus the additional sites in the extreme N-terminal segment. Expressed PR-B in Sf9 insect cells and native PR in T47D cells were uniformly labeled for 6 h with 32P, immunoprecipitated with the PR-specific monoclonal antibody AB52 (32), and digested with trypsin, and the resultant [32P]phosphopeptides were separated by HPLC using a C-18 reverse phase column.

Fig. 1 shows a tryptic phosphopeptide map of native (lower panel) and expressed PR-B (upper panel) isolated from cells that were treated with the synthetic progestin R5020 for the last 2 h of 32P-labeling. Thus, native PR was fully hyperphosphorylated (15). Native PR-B exhibits several major phosphopeptides that elute from the HPLC column between approximately 15 and 85 min (Fig. 1, lower panel). As adopted from our previous studies, each peptide has been given a number designation based on the retention time on the column. Phosphopeptide 2 is not present in the current mapping experiments, the reason being that its retention time between peaks 1 and 3 in our original studies was due to an incomplete tryptic digestion. When digested to its limit in the present study, this peptide is very small and appears in the tail through fraction with free 32P. Phosphopeptides 7 and 8 are also missing here. In our original mapping studies with native PR, we obtained three closely eluted phosphopeptides designated 7, 8, and 9 that have since been determined to represent heterogenous elution of the same phosphopeptide which we now refer to collectively as peptide 9. The closely spaced peaks at 80-min retention time represent incomplete digestions of the same peptide 12, peak 4 results from overdigestion of peptide 6, and peaks 10 and 11 appear to be the same peptide that elutes differently (14, 15). All other HPLC peaks represent distinct phosphopeptides (14, 15). Thus there appears to be at least
have determined the percent of the total incorporated $^{32}$P that was contained in each HPLC peak and shown in Fig. 1. The percent of the total $^{32}$P counts contained in each peptide was taken as the area under each HPLC peak, and the values are averages ± S.D. from multiple independent determinations. Boldface numbers are those that exhibit a significant difference between baculovirus-expressed and native PR.

| HPLC peak/site   | Baculovirus (n = 3) | T47D (n = 4) | Percent of total $^{32}$P incorporation |
|------------------|---------------------|--------------|----------------------------------------|
| p0 (Ser$^{120}$) | 5.7 ± 0.8           | 4.5 ± 0.8    |                                        |
| p1               | 20.2 ± 0.5          | 10.2 ± 1.7   |                                        |
| p3 (Ser$^{81}$)  | 4.4 ± 1.0           | 9.2 ± 1.5    |                                        |
| p4 (Ser$^{162}$) | 11.2 ± 4.4          | 6.5 ± 3.3    |                                        |
| p5               | 10.6 ± 4.9          | 8.0 ± 1.2    |                                        |
| p6 (Ser$^{162}$) | 14.3 ± 3.7          | 13.7 ± 1.5   |                                        |
| p9 (Ser$^{345}$) | 6.9 ± 1.0           | 16.5 ± 2.4   |                                        |
| p10              | 8.9 ± 3.4           | 13.1 ± 4.8   |                                        |
| p11              | 5.0 ± 0.5           | 5.4 ± 0.8    |                                        |
| p12 (Ser$^{204}$)| 12.0 ± 3.4          | 14.4 ± 4.2   |                                        |

* Phosphopeptide 4 is an overdigestion of peptide 6 and thus contains the same phosphorylation site Ser$^{162}$. Summation of the counts in peptides 4 and 6 shows little difference between baculovirus-expressed and native T47D PR.

nine different major (including phosphopeptide 2 in the fall through of the HPLC column) phosphopeptides isolated from native PR-B. We have reported on the identification of the phosphorylated residue in five of these phosphopeptides. As indicated in Fig. 1, this includes Ser$^{81}$, Ser$^{102}$, Ser$^{162}$, Ser$^{204}$, and Ser$^{345}$ which are contained, respectively, in peptides 3, 0, 6, 12, and 9. There are four additional sites that so far have been identified as HPLC isolated phosphopeptides: 1, 2, 5, and 10/11.

The HPLC phosphopeptide map of baculovirus-expressed PR-B (Fig. 1, top panel) showed a strikingly similar pattern to that of native T47D receptor. To obtain quantitative values for the relative extent of phosphorylation of individual sites, we have determined the percent of the total incorporated $^{32}$P that was contained in each HPLC-eluted peptide. All major peptides, except for 1, 3, and 9, were labeled to the same relative extent in native and baculovirus-expressed receptor. Peptides 3 (Ser$^{81}$) and 9 (Ser$^{345}$) in recombinant receptor contained significantly smaller percentages of total $^{32}$P incorporation than in native receptor. In contrast, peptide 1 in recombinant PR contained a greater percentage of total $^{32}$P incorporation than in T47D receptor (Table I). As an alternative method for phosphopeptide mapping, total tryptic digests of $^{32}$P-labeled native and baculovirus-expressed PR-B were analyzed directly by electrophoresis on alkaline 40% polyacrylamide gels (Fig. 2). In previous studies, HPLC phosphopeptide peaks generated from PR-B of T47B cells were analyzed by second dimensional electrophoresis on 40% alkaline gels (14), which established the relative mobility of the major phosphopeptides. As shown in Fig. 2, this method resolved all the major phosphopeptides that separate by HPLC as well as some additional minor peptides (Fig. 2). Even under these higher resolving conditions, hormone-treated native and recombinant PR-B exhibited the same $^{32}$P-labeled tryptic peptides (Fig. 2).

Although the HPLC phosphopeptide mapping results indicated that expressed receptor was phosphorylated on the same sites as T47D receptor, we questioned whether alternate residues within the same peptide might be phosphorylated and go undetected by single dimension phosphopeptide mapping. Therefore, we further analyzed several of the major HPLC phosphopeptides from baculovirus-expressed PR-B by manual solid-phase amino acid sequencing (35). The majority of $^{32}$P was released in cycle 8 for peptide 0, in cycle 8 for peptide 3, in cycle 3 for peptide 6 (Fig. 3), and in the first cycle for peptide 12 (not shown). It should be noted that some $^{32}$P counts in peptide 3 were released in cycle 3. This was likely due to contaminating peptide 4 which overlaps peptide 3 by HPLC and has been shown previously to release in cycle 3 (14). These cycles of $^{32}$P release were the same as those obtained previously for the equivalent peptides generated from native T47D PR (14, 15). Table II is a summary of the manual Edman degradation results with baculovirus-expressed PR and, for comparison, our previously reported data for native T47D PR. Also included in Table II are the deduced amino acid sequences of each peptide and the phosphorylated serine residues.

The level of $^{32}$P-labeling of HPLC phosphopeptide 9 was too low in the baculovirus-expressed receptor to do a phosphate release analysis. Therefore, to further compare peptide 9 from native and baculovirus-expressed PR, the HPLC-eluted fractions across peak 9 were analyzed by second dimensional alkaline 40% polyacylamide gel electrophoresis. As described in our earlier studies, peak 9 resolved into four phosphopeptide bands when electrophoresed in the second dimension. The two fastest mobility bands (Fig. 4, bands 3 and 4) were shown to be phosphorylated on a single residue at Ser$^{345}$. The reason for the different mobilities of the two phosphopeptides is not known (15). The sequence of the other two phosphopeptide (Fig. 4, bands 1 and 2) is not known. They may represent variant forms of the same tryptic phosphopeptide containing Ser$^{345}$ or other as yet unidentified phosphorylation sites. Nonetheless, the same four phosphopeptides were present in HPLC peak 9 from native and

![Fig. 2. Phosphotryptic peptide mapping of baculovirus-expressed and native PR-B by gel electrophoresis.](image-url)
baculovirus-expressed receptor (Fig. 4). The only difference was the reduced level of $^{32}$P-labeling in all four peptides isolated from the recombinant receptor. With native PR we have not yet reported on identification of sites in peptides 1, 2, 5, and 10/11. However, the cycles of $^{32}$P release with two of the peptides (1, 5) were observed to be the same with native and expressed receptor. Thus it appears that baculovirus-expressed PR is phosphorylated on all the same sites as native receptor and that phosphorylation on additional peptides, or on alternate sites within the same peptide, was not observed with expressed receptor.

**Constitutive Phosphorylation of Hormone-dependent Phosphorylation Sites—**

We have determined the effect of the progesterin agonist R5020 and two types of progesterin antagonists, RU486 and ZK98299 (41), on the total incorporation of $^{32}$P into baculovirus-expressed PR and on the phosphorylation of specific sites. To normalize $^{32}$P incorporation to PR protein, matched cultures were labeled under identical conditions with $[^{35}]$S-methionine. Both $^{32}$P- and $^{35}$S-labeled PR-B were immunoprecipitated from cell extracts with the AB52 monoclonal antibody (32), and the immunoprecipitates were resolved by electrophoresis on SDS gels and detected by autoradiography. As shown in Fig. 5, the amount of $^{32}$P-labeled baculovirus-expressed PR-B increased after treatment with hormone agonist or antagonists. However, treatment with each of three

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**Table II**

Comparison of sites of phosphorylation of native versus baculovirus PR

The phosphorylated serine residues in five of the major HPLC eluted tryptic phosphopeptides from native PR-B have previously been identified (14, 15). The cycle at which $^{32}$P was released, the amino acid sequence, and the underlined phosphorylated serine are shown in the first four columns from the left. The cycle of $^{32}$P release of tryptic phosphopeptides from baculovirus-expressed PR-B was determined by manual Edman degradation and is shown in the far right column.

| Peptide no. | Native PR $^{32}$P release cycle | Peptide sequence | Site | Baculovirus PR $^{32}$P release cycle |
|-------------|---------------------------------|-----------------|------|--------------------------------------|
| 0           | 8th                             | GAGGSSSPDFPPK   | Ser102 | 8th                                 |
| 3           | 8th                             | TDGGQSLGVEGAYSR | Ser81  | 8th                                 |
| 6           | 3rd                             | VLSPLMSR       | Ser162 | 3rd                                 |
| 9           | 2nd                             | SFCASPSVFVDFPDCAYPDPAEPK | Ser345 | ND*                                 |
| 12          | 1st                             | PLATTVMDFIHVPILPNNHALLAAR | Ser294 | 1st                                 |

*Not determined.*

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2 Y. Zhang, C. A. Beck, D. P. Edwards, and N. L. Weigel, unpublished results.
ligands also resulted in an increased synthesis and recovery of PR-B protein as indicated by the increased $^{35}$S-labeling (Fig. 5, middle panel). The increase in expression of PR in response to progesterin agonist addition to SF9 cells was observed in an earlier study (27). That progesterin agonists have a similar effect has not been reported before. The mechanism responsible for the apparent increased expression of PR is not known, but is likely due to ligand stabilization of expressed receptor protein. When the ratios of $^{32}$P to $^{35}$S counts were calculated to normalize $^{32}$P incorporation to PR protein, little or no increase in $^{32}$P incorporation per molecule of baculovirus-expressed PR-B was detected in response to any of the ligands (Table III). In contrast, we have shown previously that R5020 and RU486 produce a 2-3-fold increase (ZK98299, 80 nM RU486, or 80 nM R5020). Radioactivity in cell lysates was immunoprecipitated and subjected to SDS-PAGE and autoradiography. The right panel shows $^{32}$P incorporation into PR-A and PR-B from T47D cells that were treated with or without R5020 (40 nM).

![Fig. 5. Effects of a progesterin agonist (R5020) and two antagonists (RU486 and ZK98299) on overall $^{32}$P incorporation into PR and on synthesis of baculovirus-expressed PR. SF9 insect cells expressing PR-B were incubated for the last 6 h of viral infection with $^{32}$P orthophosphate (left panel) or $^{35}$S-methionine (middle panel). The cells were either left untreated (−) or were treated for the final 2 h of labeling with 500 nM ZK98299 (ZK986), 80 nM RU486, or 80 nM R5020. Radioactivity in cell lysates was immunoprecipitated and subjected to SDS-PAGE and autoradiography. The right panel shows $^{32}$P incorporation into PR-A and PR-B from T47D cells that were treated with or without R5020 (40 nM).](image)

**Fig. 5.** Effects of a progesterin agonist (R5020) and two antagonists (RU486 and ZK98299) on overall $^{32}$P incorporation into PR and on synthesis of baculovirus-expressed PR. SF9 insect cells expressing PR-B were incubated for the last 6 h of viral infection with $^{32}$P orthophosphate (left panel) or $^{35}$S-methionine (middle panel). The cells were either left untreated (−) or were treated for the final 2 h of labeling with 500 nM ZK98299 (ZK986), 80 nM RU486, or 80 nM R5020. Radioactivity in cell lysates was immunoprecipitated and subjected to SDS-PAGE and autoradiography. The right panel shows $^{32}$P incorporation into PR-A and PR-B from T47D cells that were treated with or without R5020 (40 nM).

![Table III. Ligand effects on overall phosphorylation of native and baculovirus-expressed PR.](image)

| Treatment | Baculovirus PR-B fold increase of $^{32}$P/$^{35}$S ratio | n* |
|-----------|-----------------------------------------------------|----|
| None      | 1.00                                                | 5  |
| ZK98299   | 0.9 ± 0.3                                           | 4  |
| RU486     | 1.0 ± 0.4                                           | 4  |
| R5020     | 1.1 ± 0.4                                           | 5  |

*Number of experiments.

R5020 in T47D cells. Although not evident from Fig. 5 because native and recombinant PR were electrophoresed on different gels, we have shown previously that the electrophoretic mobility of baculovirus-expressed PR-B bound to R5020 is indistinguishable from that of unliganded or nonupshifted native PR-B (27). The reason for this appears to be due to the fact that phosphorylation of Ser$^{345}$ of HPLC peptide 9 alone is associated with the upshift of native PR (15) and that this site is minimally phosphorylated in both unliganded and hormone-treated baculovirus-expressed PR (Fig. 4). Thus, we conclude that binding of hormone agonist or RU486 to baculovirus-expressed PR did not result in an increase in phosphate content or upshift in electrophoretic mobility as occurs with native PR.

We also performed HPLC tryptic phosphopeptide mapping of expressed PR-B isolated from SF9 cells that had been treated with or without hormone (R5020). In the absence of hormone, peptides 0 and 12 that contain Ser$^{102}$ and Ser$^{294}$, respectively, were phosphorylated to the same extent as after hormone treatment (Fig. 6). In contrast, phosphorylation of these sites in native PR was undetectable in the absence of hormone and required induction by hormone (15). Additionally, Fig. 6 shows that all other sites in baculovirus-expressed PR-B were phosphorylated to the same relative extent in the presence and absence of hormone. From the data in Fig. 6, and from two other independent experiments, we have quantitated the percent of total incorporated $^{32}$P contained in each of the major phosphopeptides as the percent area integrated under the major HPLC peaks. This analysis revealed that hormone treatment did not significantly change the relative proportion of phosphorylation of any sites in the baculovirus-expressed PR (data not shown). In contrast, similar analysis of native PR in T47D cells showed three sites at Ser$^{102}$, Ser$^{294}$, and Ser$^{345}$ that were increased substantially relative to all other sites in response to hormone (15). Thus with the exception of Ser$^{81}$ and Ser$^{345}$ which are under phosphorylated under all conditions, it appears that baculovirus-expressed PR is in the hyperphosphorylated state in the absence of hormone.

**Comparison of the Phosphate Content of Recombinant and Native Human PR—** We questioned whether the lack of hormone-stimulated phosphorylation of recombinant PR could be due to different stoichiometries of phosphorylation of the expressed and native PR. It is possible that only a small proportion of overexpressed PR becomes phosphorylated and is not representative of the total cellular pool of receptor. To determine the phosphate content of baculovirus-expressed receptor, we first measured the specific radioactivity of cellular ATP pools under our labeling conditions. These values were used to convert disintegrations/min of $^{32}$P incorporated in PR into moles of phosphate (see “Experimental Procedures”). The number of receptor molecules that were immunoprecipitated from
cell extracts was estimated by steroid binding assay. The calculated phosphate content of native PR-B in T47D cells was determined to be 4.4 phosphates per receptor in the absence of hormone, and this value approximately doubled to 10.9 phosphates per receptor after treatment with R5020 (Table IV). A similar doubling of phosphate per receptor molecule was obtained by treatment with RU486, whereas ZK98299 had little effect (Table IV). Thus for a protein with nine different phosphorylation sites, these results indicate that native PR is approximately 50% phosphorylated in the absence of ligand and becomes nearly 100% phosphorylated after hormone treatment. These estimates also substantiate our previously reported 2–3-fold increase in steady-state phosphorylation by treatment of T47D cells with R5020 (16) and the differential effect of the two antagonists on PR phosphorylation. RU486 stimulated an increase in total phosphorylation of native PR equal to that of R5020, whereas ZK98299 produced a minimal increase (16). When the same method was used to estimate the phosphate content of baculovirus-expressed PR, we obtained a value of 3.4 phosphates per receptor (PR-B) in the absence of ligand, which is comparable to that of unliganded native PR (Table IV). However, the phosphate content of baculovirus-expressed PR was not significantly affected by treatment with hormone agonist or either of the two antagonists. Thus, in the hormone-treated state, the phosphate content of baculovirus-expressed PR remained close to that of unliganded native PR.

As an independent method for measuring the phosphate content of native PR, we labeled T47D cells with [35S]methionine and converted counts of incorporated [35S] into picomoles of receptor based on determination of the specific radioactivity of total cellular [35S]-labeled protein pools (described under “Experimental Procedures”). As shown in Table IV, this method gave values for the phosphate content of native PR similar to that obtained by using steroid binding to quantitate PR. The close agreement between the two methods confirms the use of the steroid binding assay to obtain a reasonably close estimate of the stoichiometry of receptor phosphorylation. The [35S]-labeled protein pool method was not used to estimate the phosphate content of baculovirus-expressed PR for the reason that expressed PR is the major protein being synthesized at late stages of viral infection of insect cells. Thus determination of

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**Table IV**

Stoichiometry of phosphorylation of native and baculovirus-expressed PR

| Treatment                  | Steroid binding | [35S]Protein pool | Baculovirus PR-B steroid binding |
|----------------------------|-----------------|-------------------|---------------------------------|
| No hormone                 | 4.4 ± 0.3 (3)   | 4.1 ± 0.8 (3)     | 3.4 ± 1.7 (5)                   |
| ZK98299                    | 5.3 ± 0.4 (2)   | 4.8 ± 0.2 (2)     | 4.1 ± 1.2 (5)                   |
| RU486                      | 11.1 ± 4.6 (2)  | 8.5 ± 3.4 (2)     | 3.8 ± 2.1 (5)                   |
| R5020                      | 10.9 ± 2.6 (3)  | 8.3 ± 2.1 (3)     | 3.9 ± 2.9 (5)                   |

*The phosphate content of PR-B from T47D and Sf9 cells was determined by measuring the incorporation of [32P] into immunoprecipitated PR and calculating the molar ratio of phosphate to receptor. The molar amount of receptor was determined in parallel unlabeled cultures by steroid binding assay as described under “Experimental Procedures.” To determine moles of phosphate, the specific activity of the cellular ATP pool was measured and these values were used to convert incorporated [32P] disintegrations/min into moles of phosphate. The values in the table represent moles of phosphate incorporated per mole of PR-B and are averages from multiple independent determinations (n).*

*As an independent method to measure the stoichiometry of phosphorylation of native PR in T47D cells, the moles of PR present in immunoprecipitates was estimated by steady-state labeling with [35S]methionine and then converting [35S] counts incorporated in PR into moles of receptor protein based on determination of the specific activity of total cellular [35S]-labeled protein pools.*
specific activity of total \(^{35}\text{S}\)labeled protein pools under these conditions is not valid.

Baculovirus-expressed PR Fails to Associate with the Same Cytosol Oligomeric Complex as Native PR—in the absence of hormone, native PR in cytosol of target cells assemble into 9–10 S oligomeric complexes that contain heat shock protein 90 (hsp90), hsp70, p59, and possibly other cellular factors (39, 42–44). Hormone binding and treatment with salt (0.4 M NaCl) dissociates the complex resulting in a conversion of PR to a smaller 4 S sedimenting form. We questioned whether the constitutive phosphorylation of hormone-dependent sites (Ser\(^{102}\), Ser\(^{294}\)) in baculovirus-expressed PR might be due to an altered ability of expressed PR to associate with hsp90 and other cellular factors. Expressed and native PR were prepared in low ionic strength buffers as cytosolic fractions of nonhormone treated cells. Receptors were bound to \(^{3}H\)R5020 at 0–4°C and sedimented on sucrose density gradients containing sodium molybdate to stabilize the oligomeric complexes in vitro. Baculovirus-expressed PR sedimented at a smaller S value than native PR (Fig. 7). In replicate independent experiments, native PR consistently sedimented at 9–10 S while expressed PR varied between 5 and 7 S. In contrast both native and expressed PR sedimented at 4 S when treated with 0.4 M NaCl (Fig. 7). Thus, expressed PR does not appear to associate with the same cytosolic oligomeric complex as native PR. These results are consistent with the conclusion that the failure of expressed PR to assemble into a native oligomeric complex may unmask and promote phosphorylation of certain sites in the absence of hormone.

DISCUSSION

The phosphorylation state of human PR expressed in the baculovirus insect expression system was analyzed by a strategy developed previously to identify phosphorylation sites in the native receptor in T47D breast cancer cells (14, 15). Based on HPLC phosphopeptide mapping and peptide sequencing by manual Edman degradation, we report here that expressed and native PR are phosphorylated on the same sites. No alternative or inappropriate sites of phosphorylation were detected in the expressed PR. Although phosphorylation occurred only on correct sites, the phosphorylation state of expressed PR was not identical to that of native PR. The relative proportions of phosphorylation of three sites were different with expressed receptor and hormone had no effect on the phosphorylation of any sites. Two sites, Ser\(^{345}\) and Ser\(^{435}\), were proportionally less phosphorylated in the expressed PR as compared to native PR, while a third site (phosphopeptide 1) was phosphorylated to a relatively greater extent. With native PR, hormone stimulated a 2–3-fold increase in incorporation of \(^{32}\text{P}\)phosphate per receptor molecule (Table IV) which is the combined effect of a generalized increase in \(^{32}\text{P}\)-labeling of basally phosphorylated sites and induction of three major new sites at Ser\(^{102}\), Ser\(^{294}\) and Ser\(^{345}\) (15). With baculovirus-expressed PR the three hormone-induced sites were constitutively phosphorylated and showed no further increase after hormone treatment. Additionally, hormone had no effect on the extent of phosphorylation of any of the other basal sites in the expressed PR. Thus it appears that baculovirus-expressed PR is in the hormone-stimulated hyperphosphorylation state in the absence of ligand.

The reduced phosphorylation of Ser\(^{345}\) in the expressed receptor, as compared to native PR, is of interest because it provides further evidence that this site is the major determinant for the characteristic electrophoretic upshift of PR that occurs in response to hormone treatment of T47D cells (13, 16). We showed previously by phosphopeptide mapping of native PR that the only difference between upshifted and nonupshifted receptor was the presence and absence, respectively, of phosphopeptide 9 (15). The fact that hormone had no effect on the electrophoretic mobility of baculovirus-expressed PR taken together with a reduced phosphorylation of peptide 9, further supports the idea that this site is primarily responsible for the upshift. This site is also of potential importance because it appears to be both hormone and DNA binding-dependent (17).

Only one other study to our knowledge has mapped phosphorylation sites of a steroid receptor expressed in the baculovirus system. As compared with native ER in MCF-7 breast cancer cells, baculovirus-expressed ER was found to be phosphorylated only on correct sites (10, 31). However, similar to the present study with PR it appeared that some authentic sites exhibited reduced phosphorylation in the baculovirus-expressed ER. The influence of hormone on phosphorylation of baculovirus-expressed ER was not reported. Phosphorylation has been reported for some steroid hormone receptors expressed in other heterologous systems. The chicken progesterone receptor expressed in yeast was phosphorylated on the same sites that are phosphorylated on native PR in chicken oviduct. The major phosphorylation site in the vitamin D receptor was identified using vitamin D receptor overexpression in yeast and COS cells (45, 46). Three sites in the androgen receptor were identified by expression in COS cells (47), and certain sites in human ER expressed in COS cells were identified by a strategy of peptide mapping of deletion and site-directed ER mutants. However, site-specific phosphorylation may not always be identical for the same receptor expressed in different cells. For example, a major site of phosphorylation of human ER at Ser\(^{307}\) (identified in native and baculovirus-expressed ER) was not detected when ER was expressed in COS cells (9, 48).

Whether minor differences in site-specific phosphorylation of expressed steroid hormone receptors affects functional properties is not known. We have found that PR in baculovirus system

![Fig. 7. Baculovirus-expressed and native PR in T47D cells form different cytosol oligomeric complexes](Image 19553)
exhibits steroid binding and DNA binding properties that are similar to that of native PR, but we have not directly compared the transcriptional activity or other properties of native and baculovirus-expressed PR. Since there is good evidence now that phosphorylation can modulate transcriptional activity, it is possible that reduced levels of phosphorylation of Ser\textsuperscript{81} and Ser\textsuperscript{205} in baculovirus-expressed PR may affect transcriptional activity.

This study has also reported for the first time on the stoichiometry of phosphorylation of native and recombinant PR. Native PR in T47D cells was approximately 50% phosphorylated in the absence of hormone and became nearly 100% phosphorylated after hormone treatment. The only other study to report on the phosphate content of a native steroid receptor was that with the mouse glucocorticoid receptor (GR) expressed in WEHI-7 mouse thymoma cells. In the hormone-treated state, GR was phosphorylated on at least seven different sites (49) and the phosphate content was determined to be five per receptor molecule (50). Thus hormone-treated mouse GR appears to be greater than 70% phosphorylated. These results taken together with the present PR data suggest that steroid receptor phosphorylation in target tissues may be fairly homogeneous with most of the receptor molecules exhibiting phosphorylation on most sites. In the unliganded state the phosphate content of baculovirus-expressed PR was not dramatically lower than that of native PR in T47D cells. Recombinant PR contained 3.4 phosphates per receptor, while native PR contained 4.4 phosphates per receptor. Thus, overexpression of PR in insect cells did not appear to dramatically exceed the available protein kinases that phosphorylate receptor. A larger difference in phosphate content was detected after hormone treatment where native PR contained 9–10 phosphates per receptor and baculovirus-expressed PR contained about 4 phosphates per receptor (Table IV).

To what extent these small differences in phosphorylation of baculovirus-expressed PR affects its suitability for structural studies remains to be determined. The fact that phosphorylation occurs only on correct sites and with a stoichiometry that approaches that of native PR suggests that baculovirus will be an excellent source of overexpressed full-length human PR for x-ray crystallography studies. As a practical matter, phosphorylation approaches that of native receptors. Only minor differences that overexpressed PR in the baculovirus system is phosphorylated only on the same sites as native PR and that the stoichiometry of phosphorylation in T47D cells. Recombinant PR contained 3.4 phosphates per receptor, while native PR contained 4.4 phosphates per receptor. Thus, overexpression of PR in insect cells did not appear to dramatically exceed the available protein kinases that phosphorylate receptor. A larger difference in phosphate content was detected after hormone treatment where native PR contained 9–10 phosphates per receptor and baculovirus-expressed PR contained about 4 phosphates per receptor (Table IV).

Native steroid hormone receptors in the absence of ligand associate with oligomeric complexes that contain heat shock protein 90 (hsp 90) hsp70, p59, and other cellular factors that are required for assembly. These receptor-associated proteins are important for maintaining the correct folding of receptors to receive and respond to the steroid hormone. Additionally, DNA binding and transcriptional activation are linked to dissociation of receptors from the complex indicating that these associated proteins also prevent receptor from interacting productively with DNA in the absence of ligand (43, 51). The ability of steroid receptors expressed in insect cells to associate with these proteins appears to be compromised. Alnemri and Litwack (52) reported that only a small fraction of overexpressed GR was able to assemble into cytosolic oligomeric complexes; the majority of GR was expressed as an insoluble aggregate. This was not due to GR overexpression exceeding the available hsp90 or hsp70 in the insect cell. Coexpression of mammalian hsp90 and hsp70 did not increase the fraction of GR able to assemble into oligomeric complexes suggesting that other cellular factors in insect cells are either limiting or homologous enough with the mammalian equivalent to interact with steroid hormone receptors. In contrast, the majority of PR is expressed in a soluble form in insect cells (27), but it does not form the same oligomeric complex as native PR in T47D cells. This was evidenced by the substantially slower sedimentation rate of expressed cytosol PR on sucrose density gradients. Additionally, we observed by communoprecipitation of cytosolic PR that the baculovirus-expressed receptor contained a much lower amount of associated hsp90 than native PR ((27) and data not shown). We did not compare expressed and native cytosolic PR for other associated proteins. The inability of baculovirus-expressed PR to form the same oligomeric complexes as native PR did not appear to be due simply to overexpression. The same results were obtained when we lowered the level of PR expression to that of native PR (not shown).

The mechanism by which hormone stimulates phosphorylation of PR is not known. This could be due to induction of protein kinases, a change in the conformation of the receptor as a substrate for specific kinases or to dissociation of receptors from hsps resulting in unmasking of phosphorylation sites or in changing the intracellular location of the receptor. The fact that baculovirus-expressed PR was constitutively hyperphosphorylated and did not form the same association with other proteins as native PR is consistent with the conclusion that hormone-dependent phosphorylation is due to receptor dissociation from the oligomeric complex and unmasking of sites, as opposed to ligand inducing a conformational change in the receptor. Alternatively, the different association of PR with hsps may alter the intracellular location of PR in the absence of hormone and its accessibility to protein kinases.

In summary, this study shows that human PR expressed in the baculovirus insect system is phosphorylated only on the same sites as native PR and that the stoichiometry of phosphorylation of PR is not known. This could be due to induction of protein kinases, a change in the conformation of the receptor as a substrate for specific kinases or to dissociation of receptors from hsps resulting in unmasking of phosphorylation sites or in changing the intracellular location of the receptor. The fact that baculovirus-expressed PR was constitutively hyperphosphorylated and did not form the same association with other proteins as native PR is consistent with the conclusion that hormone-dependent phosphorylation is due to receptor dissociation from the oligomeric complex and unmasking of sites, as opposed to ligand inducing a conformational change in the receptor. Alternatively, the different association of PR with hsps may alter the intracellular location of PR in the absence of hormone and its accessibility to protein kinases.

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