Two Types of Anti-progestins Have Distinct Effects on Site-specific Phosphorylation of Human Progesterone Receptor*

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Human progesterone receptor (PR) is phosphorylated on multiple serine residues; three sites (Ser102, Ser294, and Ser345) are inducible by hormone agonist, while at least six others are basally phosphorylated and exhibit a general increase in response to hormone. In this study we have used high performance liquid chromatography phosphopeptide mapping and manual peptide sequencing to investigate how two different progesterin antagonists, RU486 and ZK98299, affect site-specific phosphorylation of PR isolated from T47D breast cancer cells. As compared to the progestin agonist R5020, RU486 stimulated about a 5-fold increase in overall incorporation of [32P]phosphate per PR molecule (2.5-2.6-fold for PR-A and 2.1-fold for PR-B), and at the site-specific level, RU486 stimulated both the basal and inducible sites to the same extent as R5020. In contrast, ZK98299 produced only a minimal increase in overall phosphorylation (1.2-fold for PR-A and 1.1-fold for PR-B) which was due to a reduced stimulation of the basal sites and failure to induce any of the three hormone-dependent sites. No inappropriate phosphorylation sites were detected in response to either RU486 or ZK98299. In cotreatment studies, ZK98299 blocked the increase in overall phosphorylation of PR induced by R5020, demonstrating that the failure of this antagonist to stimulate specific phosphorylation sites is not due to an inefficient interaction with PR in the intact cell. These results indicate that the biological effects of RU486 are not mediated by an alteration in the phosphorylation state of PR, whereas failure to promote phosphorylation of certain sites may contribute to the antagonist action of ZK98299. Additionally, these results support the concept of two mechanistic classes of anti-progestins that affect PR differently in vivo.

All members of the steroid/thyroid hormone receptor family that have been analyzed so far are phosphoproteins, and as a general property they become hyperphosphorylated as a rapid response to binding hormone in the cell (1-7). Sites of phosphorylation have been identified for certain members of the steroid/thyroid receptor family including mouse glucocorticoid receptor (8), chicken (9, 10) and human progesterone receptor (PR) (11, 12), human estrogen receptor (13-15), androgen receptor (16), and the vitamin D receptor (17). The major targets for phosphorylation are serine residues located in the N-terminal domain that is required for maximal transcriptional activity. However, sites in the hinge and C-terminal ligand binding domain have also been identified in certain receptors, as well as a minor phosphorylation site in estrogen receptor (18, 19), suggesting that phosphorylation may serve multiple functional roles (6, 9, 10, 14, 16, 17, 20). Although the functional role for phosphorylation of steroid receptors remains poorly defined, initial site-directed mutagenesis studies have provided direct evidence that phosphorylation of certain sites is important for receptor activity. For example, substitution of a nonphosphorylatable alanine residue for Ser118 of human estrogen receptor resulted in as much as a 60% reduction in transcriptional activity (13), and substitution of alanine for Ser130 of chick PR decreased the sensitivity of the receptor to progesterone (20).

Human PR is expressed from a single gene as two distinct molecular forms, PR-A and PR-B. The B form contains an additional 164-amino acid N-terminal sequence; otherwise the two proteins are identical throughout their common N-terminal domain and DNA and steroid binding domains (21). Phosphorylation of human PR is complex. PR-B has at least nine phosphoserines N-terminal to the DNA binding region (3, 11, 12). Three sites identified as Ser81, Ser102, and Ser162 (11, 12) are contained in the unique N-terminal segment of PR-B; the remaining sites are common to PR-A and PR-B. There appears to be two classes of phosphorylation sites based on how they are affected by progestin agonists. Three sites found in Ser-Pro consensus sequences, Ser102, Ser294, and Ser345, are highly inducible and were detected as major new sites only after treatment of cells with hormone agonist. The remaining sites are basally phosphorylated in the absence of hormone and exhibit a general increase in response to hormone agonist (12). Additionally, progestin agonists such as R5020 cause human PR to exhibit a slightly slower electrophoretic mobility on SDS-gels which is associated with phosphorylation of a single site at Ser345 (12).

Steroid analogs have been developed that function as potent competitive antagonists of progesterone (22). The mechanism by which these compounds inactivate PR remains unresolved. Because progestin agonists and antagonists induce different conformational changes in the C-terminal domain of human PR, it is generally believed that an altered receptor conformation plays an important role (23-25). Additionally, it has been suggested that anti-progestins fall into two mechanistic classes. One class, represented by ZK98299 (Onapristone), has been shown in vitro to fail to induce PR association with target DNA (26, 27). Another class of compounds, which includes the performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
clinically important antagonist RU486 (Mifapristone), is capable of inducing high affinity association of PR with target DNA (28–31). Thus it is generally believed that RU486 interferes with a receptor activation step downstream of DNA binding and that ZK98299 impairs receptor binding to DNA. This hypothesis for two classes of anti-progestins is based largely on in vitro receptor DNA binding studies and has come under challenge by studies that have assessed PR binding to specific target DNA sequences in vivo (32, 33).

Previous studies have indicated that progestin antagonists affect overall phosphorylation of human PR differently than antagonists, implying that an altered phosphorylation state of PR may contribute to the antagonist activity of these compounds. RU486 was reported to stimulate as much as a 2–3-fold higher turnover of 32P incorporated into PR in T47D cells was determined (36). RU486 was reported to stimulate as much as a 2–3-fold higher phosphorylation rate of PR as compared to the progestin agonist R5020, and ZK98299 stimulated 60% less total incorporation than R5020 (3, 34). Additionally, ZK98299 failed to promote the upshift of PR mobility on SDS-gel electrophoresis that is associated with phosphorylation (34). Whether these differences in total levels of phosphorylation represent general quantitative changes at all phosphorylation sites and/or qualitative changes, including phosphorylation of inappropriate sites, has not been investigated. In this study, we have used methods developed previously for identification of PR phosphorylation sites (11, 12) to directly analyze the effects of ZK98299 and RU486 on site-specific phosphorylation of PR in T477D breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—3-H [55]R5020 (promegestone; [17α-methyl-3H]17α, 21-dimethyl-19-norgestra-4,9-diene-3, 20-one; 87 Ci/mmol), unlabeled R5020, carrier-free [32P]H3PO4 (8200–9200 Ci/mmol), and EXPRE [58]P progesterone (1125 Ci/mmol) were obtained from Du Pont (New Haven, CT). RU486 (Mifapristone) was a gift from Roussel-UCLAF (Romainville, France) and ZK98299 (Onapristone) was provided by Dr. David Henderson, Schering (Berlin). AB-52 is a mouse monoclonal immunoglobulin G produced against purified human PR that recognizes both the A and B isoforms of PR (35). Tosylationlanalyl chlomethyl ketone-treated trypsin was from Worthington. Phenylisothiocyanate and HPLC grade acetic acid were obtained from Sigma. Sequence-AL membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA).

**Cell Culture, Radiolabeling of PR, and Receptor Preparation—**T47D human breast cancer cells were cultured as described previously (36). At the time of labeling, cultures were switched to medium containing 32P-orthophosphate, the serum containing medium was removed and for 30 min. The supernatant, considered as a control, was dialyzed against KPFM or diluted 1:1 with KPFM to reduce the salt concentration before immunoprecipitation. PR phosphoprotein, 32P counts were no longer detected in immunoprecipitated PR (data not shown). Thus to achieve uniform 32P labeling of PR, T47D cells were incubated for a total of 6 h with [32P]orthophosphate.

Harvested cell pellets were lysed in KPFM buffer (50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol) containing 0.5 mM NaCl and a mixture of protease inhibitors as described previously (36). Cells lysates were centrifuged at 100,000 × g for 30 min. The supernatant, considered as a whole cell extract, was dialyzed against KPFM or diluted 1:1 with KPFM to reduce the salt concentration before immunoprecipitation.

Immunoprecipitation and Gel Purification of PR—Radiolabeled PR in whole cell extracts were immunoprecipitated by the monoclonal antibody AB-52 (35) using protein A-Sepharose as an immunobead as described previously (30, 36). Protein A-Sepharose beads were washed with KPFM containing 0.3 mM NaCl to remove nonspecifically bound protein, and immobilized receptors were eluted with 2% SDS-sample buffer and electrophoresed on 7.0% discontinuous SDS-polyacrylamide gels as described previously (30, 36). 32P-labeled receptors were detected by autoradiography of fixed gels. In phosphopeptide mapping experiments, unfixxed wet gels were exposed to x-ray film to detect the position of radiolabeled PR bands, gel pieces corresponding to PR-A and PR-B were then excised, and radioactivity was measured by Cerenkov counting. To determine the 32P counts in PR containing either PR-A or PR-B the digests were detected, and solubilized in Absolve (Du Pont NEN) for 30 min at 55 °C, and 32P was quantitated in a liquid scintillation counter.

**Tryptic Digestion and HPLC Analysis of PR—**Gel-purified receptors were extracted and digested with tosylphenylanilyl chloromethyl ketone-treated trypsin (20 μg) as described previously (11, 12). The digested tryptic peptides were transferred to a microcentrifuge tube, dried under vacuum, and redissolved in 100 μl of 50% formic acid. The [32P]peptides were then separated on a Vydac C18 reverse-phase column by a 0–45% H2O/acetonitrile gradient containing 0.1% trifluoroacetic acid. The column was eluted by a Gilson HPLC system, was run for 50 min at a flow rate of 1 ml/min. 32P-labeled peptides were detected by an in-line 0.5-μM radio-HPLC detector from IN/US Systems (Tampa, FL) using a 0.5-mol flow cell.

**Site Identification and Phosphate Release by Manual Edman Degradation—**To determine the number and position of phosphorylated residues in each of the HPLC-eluted phosphopeptides, we performed manual Edman degradation by the method of Sullivan and Wong (38). Each of the major phosphopeptide peaks collected by HPLC was dried down, dissolved in 30 μl of 50% acetonitrile, and immobilized on an arylamine-sequenon disk. An aliquot (30 μl) of the dissolved [32P]peptide was spotted on the disk and incubated at 50 °C for 5 min by placing the disk on a Mylar sheet on top of a heating block. The aqueous solvent was then evaporated, and the peptide was covalently linked to the disk by adding 5 μl of ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (10 mg/ml in 0.1 M MES, pH 5.0) and allowing the disk to incubate at room temperature for 30 min. The disk was then washed three times with methanol and subjected to Edman degradation beginning with treatment for 10 min at 50 °C with 0.5 μl of coupling reagent (methanol/water/triethylamine/pyridine: 7:1:1:1, v/v). After five washes with 1 ml of methanol, the disk was reheat to 50 °C for 5 min with 0.5 μl of trifluoroacetic acid to cleave the N-terminal residue. The trifluoroacetic acid solution was placed in a vial, and the disk was washed with 1 ml of trifluoroacetic acid and 42.5% phosphoric acid (91, v/v). The wash was combined with the trifluoroacetic acid solution, and 32P was quantitated by Cerenkov counting. The disk was then washed five times with 1 ml of methanol before the next cycle was started.

**RESULTS**

The Progestin Antagonists RU486 and ZK98299 Have Distinct Effects on Overall Phosphorylation of Human PR—In previous time course studies we have shown that the progestin agonist R5020 stimulates a sequential increase in phosphorylation of human PR in T47D cells (12, 36). Most of the net increase in 32P incorporation into PR occurs rapidly within a few minutes of hormone addition. This is followed by a slower phosphorylation that is associated with an upshift of the electrophoretic mobility of PR on SDS gels. The major difference between upshifted and nonupshifted PR is the phosphorylation of Ser405 in the upshifted band. Because PR is phosphorylated on multiple different sites, the upshift occurs with only a slight further increase in total 32P incorporation (12, 36). In the present study we examined the effects of progestin antagonists on phosphorylation of PR by incubating T47D cells for a total of 6 h with [32P]orthophosphate and adding ligand for the last 2 h of labeling. As determined in preliminary studies (see “Experimental Procedures”), these conditions result in uniform 32P labeling of PR, and this time of exposure to hormone agonist is sufficient to stimulate both stages of phosphorylation. To ex-
Influence of ZK98299 and RU486 on Individual Phosphorylation Sites—To illustrate the complexity of human PR phosphorylation and the phosphorylation sites of human PR that have been identified so far, Fig. 3 shows an HPLC phosphopeptide mapping profile for full-length PR-B after 2 h of R5020 treatment. Thus, Fig. 3 represents the fully hyperphosphorylated state of human PR. The numbering system for HPLC eluted phosphopeptides is that adapted from our earlier studies (11, 12). There are at least nine phosphopeptides detected in PR-B and three fewer in PR-A. The shaded HPLC peaks designated as 0, 3, and 6 are unique to PR-B and represent, respectively, phosphorylation at Ser102, Ser81, and Ser162 (11, 12). Peaks designated as 1, 5, 9, 10, 11, and 12 are common to both PR-A and PR-B, and the phosphoamino acid in peptides 9 and 12 have been identified, respectively, as Ser345 and Ser294 (11, 12). We have also shown that Ser102, Ser294, and Ser345 are largely hormone agonist-dependent, while the remaining sites are basally phosphorylated and exhibit a general increase in response to hormone agonist (12). Thus, hyperphosphorylation of intact PR stimulated by hormone represents both a general increase in phosphorylation of basal sites and induction of three new sites (12).

In our earlier studies, phosphopeptide 9 exhibited heterogeneous elution behavior (originally designated as peak 7, 8, and 2.1-fold, respectively, for PR-A and PR-B. In contrast, ZK98299 promoted minimal 1.2- and 1.1-fold increases in PR-A and PR-B (Table I). It is of interest to point out that total 32P-labeling of PR-B was higher than PR-A (Fig. 1 and Table I) which is consistent with the fact that PR-B contains three additional phosphorylation sites in the N-terminal segment unique to PR-B that are not present in PR-A (11, 12) (see Fig. 3). These results show that ZK98299 and RU486 have distinct effects on the overall level of phosphorylation of PR in vivo. ZK98299 Blocks Hyperphosphorylation of PR Induced by R5020—Because ZK98299 has a lower binding affinity for PR than R5020 (32, 39), we questioned whether the failure to stimulate hyperphosphorylation might be due simply to an inefficient interaction of ZK98299 with PR in vivo as opposed to actually blocking phosphorylation when bound to PR. To compensate for the lower affinity cells were incubated with a much higher concentration of ZK98299 (500 nM) than either R5020 or RU486 (80 nM). However, one cannot be certain that cellular PR was fully occupied by ZK98299 even under these conditions. Therefore, we conducted the experiment shown in Fig. 2 and presented quantitatively in Table II. T47D cells were labeled for 6 h with 32P, and during the last 2 h of labeling cells were treated either with ZK98299 or R5020 alone, or they were coculated with ZK98299 and R5020. The concentration of ZK98299 (500 nM) was the same here as in all other experiments. However, R5020 was lowered from 80 to 20 nM to maximize competition for binding to PR by ZK98299. An SDS-gel of immunoprecipitated 32P-labeled PR shows that ZK98299 effectively inhibited the hyperphosphorylation of PR stimulated by R5020 (Fig. 2). The fold increase in total 32P incorporation into PR-A and PR-B was quantitated, and the data show that R5020 stimulation of 32P incorporation into PR-B is essentially eliminated by cotreatment with a 25-fold excess of ZK98299 (Table II). It should be noted that the fold increase in overall phosphorylation of PR stimulated by R5020 alone was slightly lower here (1.79 and 1.61, respectively, for PR-A and PR-B) than in Fig. 1 and Table I because of the reduced R5020 concentration. Nonetheless, the ability of ZK98299 to effectively inhibit hyperphosphorylation induced by R5020 strongly supports the conclusion that ZK98299 binds stably to PR in vivo and that it fails to induce the same hyperphosphorylation as the agonist R5020 and the antagonist RU486.
peptide 12 appears to be due to a resistance of Lys-Ser to trypsin when the Ser residue is phosphorylated, resulting in incomplete digestion products as described by Allen (40). Peaks 10 and 11 appear to contain the same peptide, and peak 4 results from overdigestion of peak 6. All other HPLC peaks represent a distinct phosphopeptide and a single phosphorylation site. One other difference between our current and earlier phosphopeptide maps is the lack of HPLC phosphopeptide 2. In earlier studies, peptide 2 eluted between peptides 1 and 3. It has since been found to represent an incomplete digestion product, that when completely digested to its limit, is too small to be retained by the C18 column. Nonetheless, peptide 2 is a distinct phosphorylation site that is common to both A and B receptors (11, 12).

To examine the influence of progestin antagonists on individual phosphorylation sites, we have compared tryptic phosphopeptide maps of human PR isolated from cells treated with vehicle, R5020, RU486, or ZK98299. T47D cells were labeled with 32P as in Fig. 1, and PR was immunoprecipitated from cell lysates and electrophoresed on SDS-gels. PR-A and PR-B bands were excised, eluted from gel pieces, and digested with trypsin, and the tryptic phosphopeptides were then separated by HPLC on a C18 reverse-phase column. Fig. 4 shows a comparison of tryptic phosphopeptide maps of unliganded PR-A with that of PR-A isolated from T47D cells treated with vehicle, R5020, RU486, or ZK98299. Peptide 9 (Ser345) and peptide 12 (Ser294) were barely detectable in the absence of hormone (Fig. 4A) and became major sites after treatment with R5020 (Fig. 4B). In contrast, peptides 1, 5, and 10/11 were basally phosphorylated and exhibited a general increase upon addition of hormone agonist. Treatment with RU486 (Fig. 4B, upper panel) induced phosphorylation of the two hormone-dependent sites (peptides 9 and 12) and stimulated a general increase in the basal sites (peptides 1, 5, and 10/11) in a manner similar to that with R5020 treatment. In contrast, treatment with 500 nM ZK98299 (Fig. 4A) had little effect on either of the two hormone-dependent sites or on the basal sites. Thus, phosphopeptide maps of PR-A after ZK98299 treatment are more similar to that of the unliganded receptor than hormone agonist-treated receptor.

Similar studies were performed with PR-B. Shown in Fig. 5 are tryptic phosphopeptide maps of PR-B isolated from T47D cells that were treated for 2 h with vehicle, ZK98299, RU486, or R5020. PR-B contains three additional phosphorylation sites not present in PR-A, peptide 3 (Ser35) and peptide 6 (Ser102), which contain basal sites, and peptide 0 (Ser102) which contains a hormone-inducible site (Fig. 5). Treatment with RU486 in-
increased basal and hormone-inducible sites of PR-B in a manner that was also indistinguishable from that observed after R5020 treatment (Fig. 5B). ZK98299 had a minimal effect on phosphorylation of hormone-inducible and basal sites (Fig. 5A). Thus, similar to results with PR-A, RU486 had the same effect as R5020 on the phosphorylation pattern of PR-B, whereas the phosphorylation state of PR-B after treatment with ZK98299 more closely resembled that of unliganded PR-B.

Identification of PR Phosphorylation Sites after Treatment with RU486—Although the HPLC phosphopeptide mapping patterns for R5020 and RU486 treated receptor showed no obvious differences (Figs. 4 and 5), we questioned whether RU486 might stimulate phosphorylation of an alternate residue on the same peptide that would go undetected by single dimension HPLC phosphopeptide mapping. Therefore, we directly analyzed the major HPLC tryptic phosphopeptides of PR-B by manual Edman degradation to detect the cycle of 32P release. The cycle of 32P release determines the position within a peptide of the phosphorylated amino acid residue and thus can detect whether RU486 promoted phosphorylation of any inappropriate sites. Phosphate release can also distinguish between the presence of a single or multiple phosphorylated residues within a peptide. The major HPLC phosphopeptides for which we have identified sites, and their characteristic cycle of 32P release as reported in our earlier studies, are shown in Table III for PR-B isolated from R5020-treated cells. This includes peptide 0, 3, 6, 9, and 12 that each contain, respectively, phosphorylated residues at Ser102, Ser81, Ser162, Ser345, and Ser294. Fig. 6 shows the cycle of phosphate release for peptides 9 and 12 of PR-B isolated from RU486-treated cells. The major cycle of 32P in peptide 9 was released in cycle 2 and in peptide 12, the majority of 32P was released in cycle 1. Thus RU486 and R5020 stimulated phosphorylation of the same two major hormone dependent sites (Ser294 and Ser295) that are common to both PR-A and PR-B. Analysis of peptides 0, 3, and 6 also showed identical cycles of 32P release for R5020- and RU486-treated receptors (Table IV). Thus, all three PR-B-specific sites (Ser81, Ser102, and Ser162) were phosphorylated identically by R5020 and RU486. Also, we have detected no differences between R5020- and RU486-treated receptors in the other basal phosphorylation sites (not shown). Thus, we conclude that RU486 stimulated phosphorylation on all the same sites as hormone agonist and that it does not promote phosphorylation of alternate sites.

**DISCUSSION**

A potentially important question that has not been fully explored is whether the biological action of anti-progestins is mediated in part by altering the phosphorylation state of the progesterone receptor. Previous studies that examined the effects on net 32P incorporation into human PR suggested that this might be the case, since RU486 was reported to stimulate a substantially greater increase in total phosphorylation than a progesterin agonist (R5020) and ZK98299 effected a much reduced increase as compared to R5020 (3, 34). Because of the complexity of human PR phosphorylation (11, 12), it is not possible to interpret whether these differences in total phosphorylation simply represent a general quantitative change in the degree of phosphorylation of all sites or whether this reflects a more qualitative change in a few sites. It is also possible that antagonists may promote the phosphorylation of new inappropriate sites. In contrast to previous reports with human PR, we did not detect a difference between the effects of RU486 and R5020 on net 32P incorporation into PR in T47D breast cancer cells. Both ligands stimulated essentially the same 2-1-fold (PR-B) and 2.5-2.6-fold (PR-A) increase (Table I) which differed from the 3-5-fold increase for R5020 and 5-10-fold increase for RU486 reported in earlier studies (3, 34). The reason for this apparent discrepancy is not known, but is likely due to differences in methodology. In the present study we have used phosphatase inhibitors during the isolation of PR to minimize dephosphorylation during in vitro processing which was not done in earlier studies, and we have normalized 32P incorporation to PR protein by calculating the ratio of 32P to 35S counts.

As compared to the agonist R5020 we did detect a reduced stimulation of net 32P incorporation into PR after treatment with ZK98299. However, the reduction was greater than the 60% reported earlier with human PR (34). After normalization to PR protein, we found that net 32P incorporation after ZK98299 treatment was only 10% (PR-B) to 20% (PR-A) of that obtained after treatment with R5020 (Table I). Because ZK98299 has a lower affinity for PR than either progesterone or R5020 (32, 39), we questioned whether the lack of hyper-
phosphorylation could be due simply to an inefficient interaction of ZK98299 with PR in vivo. However, at concentrations where ZK98299 itself had little effect, it was able to block hyperphosphorylation of PR induced by R5020 (Fig. 2) or by RU486 (not shown). These results provide strong evidence that ZK98299 does in fact bind efficiently to PR in vivo and fails to induce hyperphosphorylation. This ability of ZK98299 to block hyperphosphorylation induced by other ligands has not been reported before.

By phosphotryptic peptide mapping and manual amino acid sequencing to determine the cycle of $^{32}$P release, we further showed in this study that R5020 and RU486 phosphorylated all the same major sites on human PR and to the same relative extent. Additionally, no alternative sites on the same peptide were phosphorylated after treatment with RU486, nor were any new inappropriate sites detected. Thus under the conditions of our assays, we conclude that RU486 and progestin agonists have indistinguishable effects, both quantitatively and qualitatively, on the phosphorylation state of human PR. Similar conclusions were drawn from earlier phosphopeptide mapping studies of human PR (3). However, identification of the amino acid residues phosphorylated after RU486 treatment was not reported, making it difficult in these earlier studies to rule out whether RU486 was promoting phosphorylation of alternative sites. Additionally, the HPLC methodology of the present study was of higher resolution, allowing the isolation of additional phosphopeptides not detected earlier (3).

Phosphopeptide mapping of human PR after treatment with

![Phosphotryptic peptide mapping of PR-A](image.png)

**Fig. 4.** The progestin antagonists RU486 and ZK98299 have distinct effects on tryptic phosphopeptide mapping of PR-A. T47D PR labeled to steady-state with $^{32}$P was immunoprecipitated, gel purified, and digested with trypsin, and the tryptic [$^{32}$P]-peptides were resolved by HPLC as in Fig. 3. Cells were treated during the last 2 h of labeling with A, vehicle or ZK98299 (500 nM); B, RU486 (80 nM) or R5020 (80 nM). The shaded peaks indicate hormone-dependent phosphopeptides.
ZK98299 has not been reported previously; only effects on net $^{32}$P incorporation have been examined. By phosphopeptide mapping and manual peptide sequencing we also determined in this study that ZK98299 failed to induce any of the three hormone agonist-dependent sites (Ser$^{102}$, Ser$^{294}$, and Ser$^{345}$). Additionally, the basal phosphorylation sites were unaffected, and no new inappropriate phosphorylation sites were detected after ZK98299 treatment (Figs. 4 and 5). Therefore, the modest increase in net $^{32}$P incorporation stimulated by ZK98299 (1.1-fold for PR-A and 1.2-fold for PR-B; Table I) would appear to reflect a slight increase in general phosphorylation of the basal sites. Interestingly, ZK98299 also failed to produce the same upshift in electrophoretic mobility as progestin agonist. This is consistent with its failure to phosphorylate Ser$^{345}$, since the upshift was shown previously to be associated with phosphorylation of Ser$^{345}$ (12).

Phosphopeptide mapping studies (without identification of the phosphorylated residues) with rabbit PR expressed in COS cells yielded somewhat different results with respect to the effects of progestin agonist and antagonists. On a similar note, both RU486 and R5020 stimulated the same fold increase in net $^{32}$P incorporation with rabbit PR (41). However, rabbit PR generated seven phosphopeptides, all of which were basally phosphorylated, and both ligands stimulated a general in-
crease in phosphorylation of all phosphopeptides in a proportionately similar manner. Thus, induction of major new phosphorylation sites by progestin agonist or RU486, as occurs with human PR in T47D cells, was not detected with rabbit PR (41). Additionally, ZK98299 effects on site-specific phosphorylation of rabbit PR differed from our results with human PR in that it produced a general decrease in phosphorylation of all basal sites. Rabbit and human PR phosphorylation may simply respond differently to ligands, or alternatively, these results may be due to differences in methods. Rabbit PR was overexpressed in COS cells by transient transfection, whereas human PR were analyzed in their natural cellular environment as an endogenous gene product. When human PR was expressed from baculovirus vectors in insect cells, we found that phosphorylation occurred on correct sites, but hormone-dependent phosphorylation was not detected.2 Thus it is possible that ligand effects on receptor phosphorylation can change when receptors are expressed in heterologous cells.

Based largely on in vitro binding studies, it has been suggested that progestin antagonists can be categorized by two mechanistic classes, those such as RU486 that promote efficient binding of PR to DNA and others such as ZK98299 that fail to do so (26, 27). However, two studies that have analyzed PR binding to specific target DNA in vivo do not support this hypothesis. Cotransfection studies that were able to detect competition for binding of PR to progesterone response elements (PREs) in vivo have suggested that both types of progestin antagonists stimulate PR-DNA binding in the intact cell (32). In contrast in vivo footprinting studies have suggested that neither RU486 nor ZK98299 are capable of promoting the binding of PR to PREs in the intact cell (33). Biological studies have suggested that RU486 and ZK98299 do in fact affect PR differently in the intact cell. For example, under conditions where ZK98299 behaves as a complete antagonist, RU486 can exhibit weak partial agonist activity (29, 42). Additionally, we and others have shown that activators of cAMP signaling pathways can promote functional switching of RU486 from that of a potent progestin antagonist to a relatively strong agonist. Under the same conditions ZK98299 continues to behave as a pure antagonist (43, 44). The present study showing that RU486 and ZK98299 have different effects on phosphorylation of PR in the intact cell further supports the concept of two mechanistic classes of anti-progestins. We conclude from our analysis of

### TABLE III
Characteristics of serine phosphorylation sites of human PR

In previous studies we identified the phosphorylated serine residues in five of the major HPLC-eluted tryptic phosphopeptides generated from PR-B of R5020-treated T47D cells (11, 12). The cycle at which $^{32}$P was released by manual Edman degradation, the amino acid sequence, and the phosphorylated residue for each of the peptides are depicted in the table. In the present study, the characteristic cycle of $^{32}$P release from each of these peptides was confirmed.

| Peptide no. | $^{32}$P release cycle | Peptide sequence | Site |
|-------------|------------------------|-----------------|------|
| 0           | 8th                    | 4HGAGGSSSSEP8E    | Ser102 |
| 3           | 8th                    | 4TDOQQLSDEVEGAYSR | Ser81  |
| 6           | 3rd                    | 160VLSPLMCSP8175  | Ser162 |
| 9           | 2nd                    | 445SSPACSTTVAVGDFPDCAYPPDAPK | Ser345 |
| 12          | 1st                    | 294SPLATTVMDFHVPLNHALLAR | Ser294 |

![FIG. 6. Manual Edman degradation to determine the position of $^{32}$P-labeling in two major tryptic phosphopeptides.](image1.png)

**TABLE IV**
Comparison of sites phosphorylated in human PR treated with R5020 or RU486

PR-B from T47D PR treated with R5020 (2 h) or RU486 (2 h) were isolated by immunoprecipitation and SDS-gels. Gel-purified PR-B was then digested with trypsin and subjected to reverse-phase HPLC on a Vydak C18 column. The indicated HPLC-eluted tryptic phosphopeptides were analyzed by manual Edman degradation. Release of $^{32}$P was determined after each cycle.

| Peptide no. | Cycle of $^{32}$P release |
|-------------|----------------------------|
| Peptide 9   | + R5020 | + RU486 |
| 0           | 8       | 8       |
| 3           | 8       | 8       |
| 6           | 3       | 3       |
| 9           | 2       | 2       |
| 12          | 1       | 1       |

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specific phosphorylation sites that RU486 promotes phosphorylation of human PR on all the same serine residues as progesterin agonists and that the antagonist activity of this compound cannot therefore be mediated by altering the phosphorylation state of the receptor. In contrast, the dramatic effect of ZK98299 on blocking hyperphosphorylation of PR suggests that the antagonist activity of ZK98299 may be mediated in part by preventing phosphorylation of sites important for receptor activation. Thus we propose that RU486 and ZK98299 inhibit PR activation by different mechanisms; RU486 antagonism does not involve alteration in receptor phosphorylation, whereas blocking of certain phosphorylation sites may be involved in the action of ZK98299.

At the present time, the functional role of phosphorylation of human PR is unknown. Thus far, only a few studies have addressed the functional role of phosphorylation of any steroid receptor directly by mutating authentic phosphorylation sites and determining the effect that lack of a specific phosphorylation has on receptor activity in vivo. Initial studies with estrogen receptor (13) and chick PR (20) have shown that substitution of a single serine phosphorylation site, among several sites, had a substantial effect on receptor activity in vivo. In general, phosphorylation of eukaryotic transcription factors is an important means for modulating activity, and like steroid receptors, other transcription factors are frequently involved in the action of ZK98299.

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