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Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: Analysis with zinc finger mutants and the progesterone antagonist ZK98299

(human progesterone receptors/DNA binding mutants/T47D breast cancer cells)

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ABSTRACT **Human progesterone receptors (hPRs) are phosphorylated at multiple serine residues, first in a basal step and then in a hormone-induced step. To determine whether hormone-induced phosphorylation precedes or follows the interaction of hPRs with DNA two strategies were used. (*i*) DNA binding was prevented or altered with site-specific mutants of the A form of hPR; (*ii*) DNA binding of wild-type hPR forms A and B was prevented with the progesterone antagonist ZK98299. Two hPR_A mutants were constructed: DBD_{Cys}, which lacks a critical cysteine residue in the first zinc finger, and DBD_{sp}, which is mutated at three discriminatory amino acids to change its DNA binding specificity from a progesterone response element to an estrogen response element. Receptors were transiently expressed in PR-negative cells and were intranuclear. DBD_{Cys} did not bind DNA *in vitro* and DBD_{sp} bound only the estrogen response element. Transiently expressed hPR_A and DBD_{sp} showed the upward shift in electrophoretic mobility characteristic of hormone-induced phosphorylation; it was absent with DBD_{Cys}. Hormone-induced [^{32}P]orthophosphate incorporation into transiently expressed DBD_{Cys} was reduced 60% compared to hPR_A and DBD_{sp} but was not eliminated. ZK98299 binds hPRs but prevents their interaction with DNA. Compared to R5020, the antagonist reduced phosphorylation of hPR_B and hPR_A in T47D breast cancer cells by 60% and totally prevented the mobility shift. We conclude that the hormone-induced phosphorylation of hPR includes DNA-independent and DNA-dependent stages and that only DNA-dependent sites contribute to the mobility shift.**

Human progesterone receptors (hPRs) consist of two naturally occurring phosphorylated proteins—B receptors (hPR_B) of 120 kDa and A receptors (hPR_A) of 94 kDa (1, 2). In T47D breast cancer cells, phosphorylation occurs at low basal levels co- and post-translationally (2), and hormone occupancy rapidly increases the specific activity of [^{32}P]orthophosphate incorporation into receptors by 3- to 5-fold over basal levels (2–7). Basal and hormone-induced phosphorylation have been mapped to 6 or 7 serine residues in the amino terminus of chicken and human receptors (8, 9). An additional hormone-induced phosphoserine residue has been localized to the hinge region of chicken PR by phosphopeptide sequencing (10). In fact, multiple-site phosphorylation may be the rule for steroid receptors, as judged by the recent identification of six phosphoserines and one phosphothreonine in mouse glucocorticoid receptors (52).

Steroid receptors are ligand-activated transcription factors (ref. 11 and references therein). Unliganded receptors are bound by accessory proteins, including heat shock proteins, that apparently repress their activity (12). Upon hormone binding some of these factors are released, the receptors dimerize (13–16), and they acquire specific DNA binding capacity (17) in a step that may also involve cooperative binding between receptor dimers (18, 19) and association of receptors with other binding proteins (20) and transcription factors (21–23). At least some of these complex structural and functional changes are likely to be linked to hormone-induced phosphorylation, judging from the role of phosphorylation in modulating the action of other transcription factors (24–27). However, evidence for functional changes resulting from phosphorylation of steroid receptors is limited. Basal phosphorylation may modulate the affinity of receptors for their ligands (28), although this is not firmly established (29). It may serve additional functions since basal phosphorylation continues long after hPRs have acquired hormone binding capacity (2). Little is known of the function of hormone-induced phosphorylation. Only its relationship to DNA binding has received much experimental attention, with results that remain inconclusive. PR binding to DNA is independent of phosphorylation according to some studies (30, 31) and is influenced by phosphorylation according to others (4).

To determine whether hormone-induced hPR phosphorylation precedes DNA binding (and perhaps influences this binding) or follows DNA binding (and influences transcription) we used two strategies. (*i*) The DNA binding ability of hPR_A was either eliminated or modified; (*ii*) the DNA binding of wild-type PR was blocked with the type I progesterone antagonist ZK98299 (32). Thus, we analyzed the relationship of phosphorylation to DNA binding by altering the DNA binding state rather than the phosphorylation state of the receptors.

The DNA binding domain (DBD) of hPRs is composed of two fingers, each formed by a zinc atom coordinated by four invariant cysteine residues (11). To eliminate the DNA binding of hPR_A, Cys^{587}, located at the base of the first zinc finger, was mutated to alanine to create DBD_{Cys}. In glucocorticoid receptors, which recognize the same DNA sequence as PR (17), mutation of the homologous residue, Cys^{460}, leads to complete loss of transcriptional activity (33). Additionally, the DNA binding specificity of steroid receptors is due to three discriminatory amino acids at the base of the first zinc finger (17, 34). Therefore, DBD_{sp} was created by mutation of Gly^{585}, Ser^{586}, and Val^{589} to glutamic acid, glycine, and alanine, in order to change the DNA binding specificity of hPR from a progesterone response element (PRE) to an estrogen response element

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Abbreviations: hPR, human progesterone receptor; DBD, DNA binding domain; PRE, progesterone response element; ERE, estrogen response element.

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(ERE). This mutant was used to determine the DNA sequence specificity requirements for PR phosphorylation.

Analysis of the mutant and wild-type A receptors expressed in COS-1 cells suggests that hormone-induced phosphorylation can be subdivided into two stages. The first stage is DNA independent, for which DBD_{Cys} is a substrate; the second is DNA dependent, for which DBD_{Cys} is not a substrate. These results were confirmed with ZK98299-occupied wild-type B and A receptors, which are only substrates for the DNA-independent stage. We propose that multiple sites in hPRs are phosphorylated sequentially in a progressive cascade.

METHODS

Site-Directed Mutagenesis. The hPR_A expression vector hPR2, cloned into pSG5 (35), was a gift of P. Chambon (Strasbourg, France). hPR2 was mutated using oligonucleotide-directed site-specific mutagenesis employing single-stranded template DNA (36). In DBD_{Cys}, Cys^{587}, located at the base of the first zinc finger, was mutated to Ala^{587}. In DBD_{sp}, the sequence Gly-Ser-Cys-Lys-Val^{589} at the base of the first zinc finger was mutated to Glu-Gly-Cys-Lys-Ala^{589}. Mutations were verified by dideoxy sequencing.

Cell Transfections. Transfections into COS-1 cells were performed by calcium phosphate coprecipitation (37) using 5 μg of the hPR expression plasmids together with 3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) to monitor transfection efficiency and 12 μg of Bluescribe carrier plasmid (Stratagene) for a total of 20 μg of DNA. Cells were plated onto 100-mm culture dishes, grown in modified Eagle's minimal essential medium supplemented with 5% fetal calf serum, and harvested 48 hr after addition of precipitated plasmid DNA. For hormone treatment, 0.1 μM progestin R5020 or anti-progestin RU486 was added to the cells 2 hr prior to harvest. Cells from duplicate dishes were pooled for preparation of whole cell extracts by homogenization in 0.6 M KCl or for separate isolation of cytosolic and 0.6 M KCl nuclear extracts as described (6).

[^{32}P]Orthophosphate Labeling and Immunoprecipitation. Transfected COS-1 cells or T47D breast cancer cells were treated with or without 0.1 μM RU486 or R5020 (Roussel-Uclaf) or with the antagonist ZK98299 or ZK112993 (Schering) and metabolically labeled with [^{32}P]orthophosphate; then labeled whole-cell 0.6 M KCl extracts prepared from duplicate culture dishes were immunoprecipitated with hPR-specific antibodies, AB-52 and/or B-30 as described (9, 38). Eluted receptors were resolved on a denaturing 7.5% polyacrylamide gel and transferred to nitrocellulose. Immunoblots were performed as described (7, 9). Receptor protein (immunoblot) and ^{32}P incorporation (autoradiogram) were quantitated by densitometry.

Gel Mobility Shift Assay. Gel mobility shift assays were performed as described (36) using nuclear extracts prepared from transfected COS-1 cells treated with R5020 for 2 hr prior to harvest. Cells from duplicate transfections were pooled for extract preparation. The following probes were employed: a 27-mer containing the PRE from the rat tyrosine aminotransferase gene promoter (39) (5′-AAAGTCTGTACAGGATGT-TCTGATCAA-3′) and a 35-mer containing the ERE from the *Xenopus* vitellogenin A2 gene promoter (40) (5′-GTC-CAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′).

Immunofluorescence. COS-1 cells were seeded onto glass coverslips in Petri dishes. Culture and transfection conditions were as described above. Cells were fixed by treatment with 0.5% paraformaldehyde, permeabilized with 0.1% Triton X-100, washed, and incubated for 60 min at 4°C with 10 μg of AB-52 per ml as described (41). The cells were washed and incubated for 30 min at 4°C with 40 μg of fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ per ml (Boehringer Mannheim). Coverslips were washed and mounted, and cells were photographed using a ×100 oil immersion objective.

RESULTS

Since the ability of hPRs to serve as accurate substrates for specific kinases may depend on their intracellular location, the expression and intracellular distribution of the wild-type and mutant receptors in transfected cells treated with R5020 were analyzed by immunofluorescence microscopy (Fig. 1) and hPR flow cytometry (not shown). Approximately 8–9% of cells express the proteins and wild-type and mutant receptors show a clear nuclear fluorescence. However, the intranuclear staining pattern of the DBD_{Cys} mutant (Fig. 1*B*), characterized by a mottled distribution, differs from the more homogenous pattern seen with the wild-type (Fig. 1*A*) and the DBD_{sp} (Fig. 1*C*) receptors. Some cytoplasmic labeling (Fig. 1*B*, right panel) is more often seen with DBD_{Cys} than with the wild-type receptors.

The DNA binding properties of the receptors are shown in Fig. 2. Transfected COS-1 cells were treated with or without 0.1 μM R5020, and hPRs were extracted and analyzed for DNA binding by gel mobility shift assay. Binding of wild-type hPR_A to the PRE (lane 1) is supershifted by the anti-PR antibody AB-52 (lane 2). As shown by Meyer *et al.* (42), hormone-free hPRs extractable from nuclei do not bind DNA (lane 3). As predicted, the two DBD mutants cannot bind a PRE (lanes 4–7), but DBD_{sp} can bind an ERE and can be supershifted by the anti-PR antibody (lanes 8 and 9). DBD_{Cys} appears to have lost all specific hormone response element binding capacity (lanes 10 and 11). In contrast to wild-type hPRs, neither mutant was able to regulate transcription of the chloramphenicol acetyltransferase gene linked to a PRE-containing thymidine kinase promoter (not shown).

On immunoblots wild-type hPR_B of T47D human breast cancer cells has a triple protein band structure and hPR_A is a singlet. When T47D cells are treated with the agonist R5020 or the antagonist RU486, rapid structural changes occur in hPRs characterized by apparent increases in the molecular weights of all protein bands accompanied by intense labeling with [^{32}P]orthophosphate (2, 9). To test the role of DNA

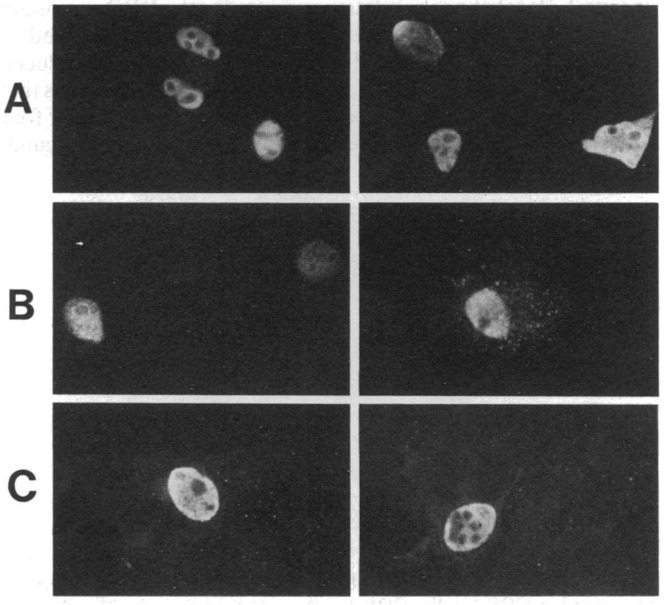

FIG. 1. Immunofluorescence analysis of COS-1 cells transiently expressing wild-type hPR_A or DBD mutant PR. Cells were transfected with hPR2 (*A*), DBD_{Cys} (*B*), or DBD_{sp} (*C*) and treated with 0.1 μM R5020 for 4 hr; they were fixed, permeabilized, and incubated with the anti-PR antibody AB-52, followed by incubation with anti-mouse fluorescein isothiocyanate-conjugated $F(ab')_2$.

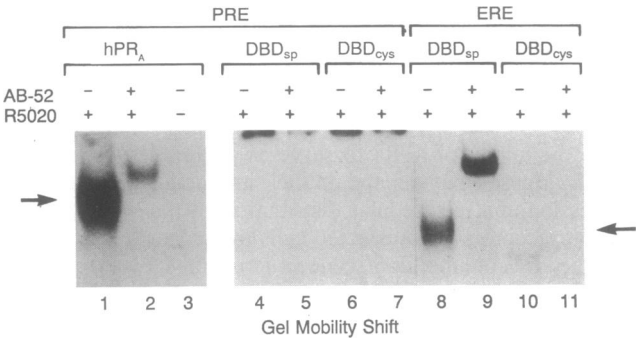

FIG. 2. Hormone-induced binding of wild-type hPR$_A$ and two hPR DBD mutants to the rat tyrosine aminotransferase PRE or the *Xenopus* vitellogenin ERE in the presence or absence of antibody AB-52. COS-1 cells were transfected with the wild-type hPR$_A$ expression vector or the DBD mutants and treated with R5020 (+) or left untreated (−); nuclear extracts were incubated with ^{32}P-end-labeled hormone response element-containing double-stranded oligonucleotides in the presence (+) or absence (−) of AB-52. Protein–DNA complexes were resolved on nondenaturing gels, dried, and autoradiographed. Arrows mark the positions of hPRs bound to DNA, which differs for the PRE and ERE. See text for lane designations.

binding on the hormone-induced electrophoretic mobility shifts, mutant and wild-type A receptors from transfected COS-1 cells were analyzed by immunoblotting (Fig. 3). The hormone-induced upward shift is seen with hPR$_A$ (compare lanes 1 and 2), but not with DBD$_{Cys}$ (lane 4), suggesting that DNA binding is required to generate this change in electrophoretic mobility. However, PRE sequence specificity is not required since DBD$_{sp}$ is shifted upward in a hormone-induced manner while bound to an ERE (compare lanes 5 and 6).

To compare the extent of hormone-dependent phosphorylation in wild-type and mutant receptors, transfected COS-1 cells were incubated with [^{32}P]orthophosphate in the presence or absence of hormone. Incorporation of ^{32}P (Fig. 4) was normalized to nuclear receptor protein levels based on densitometry of immunoblots (not shown). Basal hPR$_A$ phosphorylation in hormone-untreated cells is shown in lane 1, and the hormone-induced increase in [^{32}P]orthophosphate incorporation is shown in lane 2. Basal phosphorylation is normal in the DBD$_{Cys}$ mutant (lane 3) but the hormone-induced phosphorylation is reduced by ≈60% (compare lanes 2 and 4). In contrast, hormone-induced phosphorylation of the DBD$_{sp}$ mutant (lane 6) is the same as that of wild type (lane 2). This experiment is representative of four studies in which either R5020 or RU486 was used as the ligand.

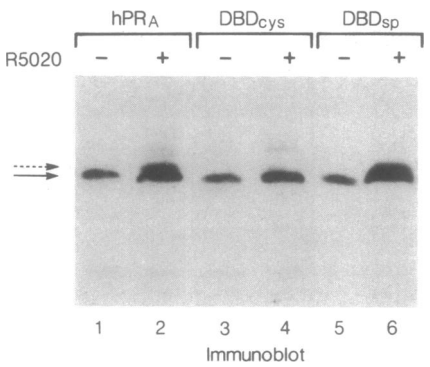

FIG. 3. Immunoblot of wild-type and two DBD mutant A receptors expressed in COS-1 cells. COS-1 cells were transfected with wild-type hPR$_A$ or the cysteine and specificity DBD mutants and treated with (+) or without (−) 0.1 μM R5020 for 2 hr before harvesting; total cellular receptors were extracted with 0.6 M KCl. Extracts were resolved by electrophoresis, transferred to nitrocellulose, and immunoblotted with antibody AB-52. The band that is shifted upward is marked by the dashed arrow. See text for lane designations.

Results were qualitatively similar, although the intensity of hormone-induced phosphorylation is greater when PRs are bound by RU486 (see Fig. 5), as we have previously reported (9). These data suggest that a fraction of total hormone-induced phosphorylation of hPRs is DNA independent and that the remainder is dependent on the binding of hPRs to DNA. The DNA need not be a PRE; an ERE will suffice. Taken together with the data in Fig. 3, we also conclude that the upward shift in electrophoretic mobility seen after hormone treatment of A receptors is due to sites phosphorylated in the DNA-dependent stage.

Since the hPR phosphorylation machinery of COS-1 cells, which are derived from monkey kidneys, differs subtly from that of human breast cancer cells (not shown), an alternate strategy was used to analyze the role of DNA binding on phosphorylation of hPR$_A$ and hPR$_B$ in human cells. Recently, Klein-Hitpass *et al.* (32) reported that the progesterone type I antagonist ZK98299 prevents hPR binding to the PRE of the ovalbumin promoter, whereas the type II antagonists RU486 and ZK112993 and the agonist R5020 promote such binding. Our studies indicate that hPRs occupied by ZK98299 intracellularly bind a synthetic palindromic PRE *in vitro* at a level <5% that of PRs occupied by the other progestin agonists and by type II antagonists (not shown). In T47D cells, ZK98299 binds hPRs and fully antagonizes the agonist effects of R5020 on a stably transfected mouse mammary tumor virus PRE linked to chloramphenicol acetyltransferase (not shown). T47D cells were therefore treated with the agonists or antagonists, and hPR structure and phosphorylation were analyzed by immunoblotting and [^{32}P]orthophosphate incorporation (Fig. 5). The immunoblot (Fig. 5 *Left*) shows the characteristic triplet B, singlet A structure of hormone-unoccupied wild-type hPRs (lane 1) and the molecular weight upward shifts of hPR$_A$ and hPR$_B$ associated with phosphorylation (2) that are induced by R5020 (lane 2), RU486 (lane 3), and ZK112993 (lane 5). This upward shift is arrested by ZK98299 occupancy (lane 4). The autoradiogram (Fig. 5 *Right*) shows the basal phosphorylation state of hormone-unoccupied receptors (lane 1) and the hormone-induced phosphorylation produced by R5020- (lanes 2a and 2b), RU486- (lane 3), and ZK112993- (lane 5) occupied receptors.

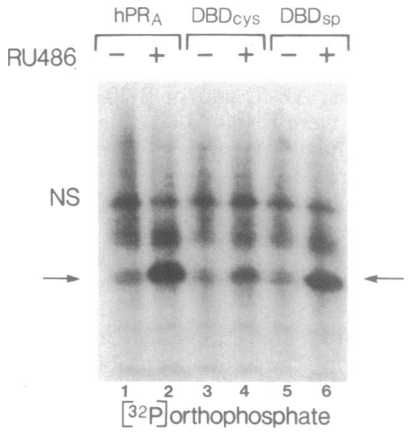

FIG. 4. Comparison of the extent of [^{32}P]orthophosphate incorporation into wild-type and two DBD mutant A receptors. COS-1 cells transfected with hPR$_A$ or the two DBD mutants and cotransfected with a β-galactosidase expression vector were preincubated with phosphate-free medium and then pretreated with (+) or without (−) 0.1 μM RU486 before the cells were labeled 4 hr with [^{32}P]orthophosphate. Aliquots of nuclear extracts were immunoprecipitated with AB-52, solubilized, resolved by electrophoresis, transferred to nitrocellulose, and autoradiographed. ^{32}P incorporation into PRs was normalized to receptor protein levels obtained from immunoblot analysis (not shown). The position of hPR$_A$ is marked by the arrows. See text for lane designations.

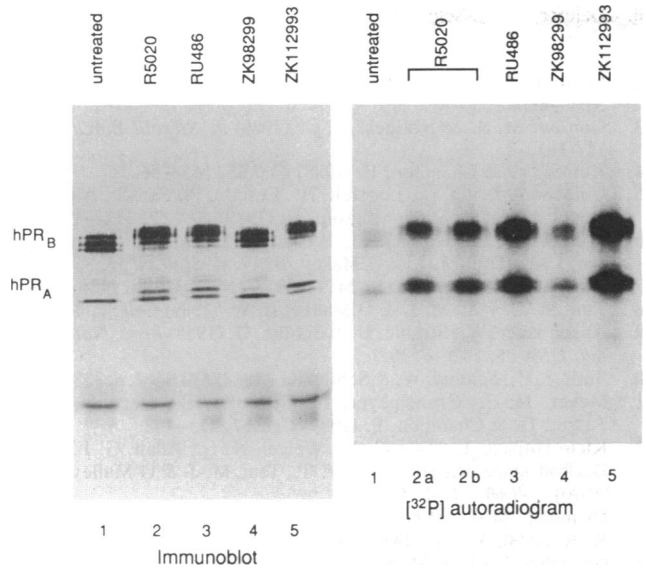

FIG. 5. Antiprogestin ZK98299, which prevents hPR binding to DNA, arrests the hormone-induced molecular weight upward shifts and underphosphorylates wild-type PRs. T47D cells were incubated without or with 0.1 μM progestins and [^{32}P]orthophosphate for 4 hr. Whole-cell extracts were immunoprecipitated with B-30 and AB-52 (*Left*) or AB-52 (*Right*), eluted, and resolved by electrophoresis. The proteins were transferred to nitrocellulose and the sheet was probed with AB-52 to generate the immunoblot. The nitrocellulose sheet from a separate experiment was exposed to x-ray film to obtain the ^{32}P autoradiogram. See text for lane designations.

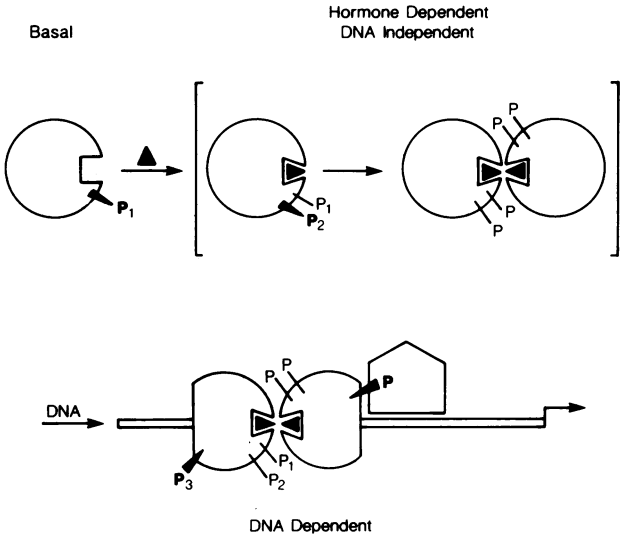

FIG. 6. Model describing a three-stage hPR phosphorylation cascade. Newly synthesized receptors are phosphorylated at basal sites, P_1. Hormone binding (solid triangle) leads to allosteric structural modifications that expose new sites to phosphorylation, P_2, while the receptors are in solution. This DNA-independent phosphorylation is rapid and may precede (as shown) or follow (not shown) receptor dimerization. This stage is transient (hence, the brackets) and quickly leads to DNA binding, which promotes additional conformational changes of the receptors. This exposes new sites, P_3, to different kinases that catalyze the DNA-dependent phosphorylation. The DNA-bound, fully phosphorylated PR dimers interact with other proteins on a promoter to regulate transcription.

Compared to the agonist R5020, the two type II progesterone antagonists, which promote PR binding to DNA, hyperphosphorylate the receptors (compare lane 2 with lanes 3 and 5). Phosphorylation is increased 3.7-fold above basal by R5020, 5.1-fold above basal by RU486, and 8.5-fold above basal by ZK112993. In contrast, the ZK98299-occupied PRs are underphosphorylated, but phosphorylation is still increased 1.5-fold above basal. Compared to R5020, phosphorylation of ZK98299-occupied receptors is 60% reduced. This study confirms the DNA dependence of the phosphorylation that produces the molecular weight upward shift and demonstrates that a fractional component of hormone-induced phosphorylation is DNA independent.

DISCUSSION

The increasing evidence that PRs are phosphorylated at multiple serine residues has hinted at complex regulatory functions for this covalent modification. Based on the data described here, we propose that hPR phosphorylation occurs in a progressive, three-stage cascade depicted in the model shown in Fig. 6. First, in the basal stage (P_1), low levels of phosphate are incorporated into receptors cotranslationally, which continues posttranslationally in a slow maturation step that may take 6–10 hr (2). Second, hormone occupancy (shown by the solid triangle) leads to allosteric changes that expose additional serine residues to phosphorylation. This second phosphorylation stage (P_2) is extremely rapid since it occurs within 10 min of hormone treatment (1); it is accompanied by receptor dimerization but precedes DNA binding (13–15). This hormone-dependent, DNA-independent stage accounts for a fraction of total hormone-dependent phosphorylation, as shown by the residual phosphorylation of the DBD_{Cys} mutant and the ZK98299-occupied PR. The third phosphorylation stage (P_3) depends on DNA binding. We postulate that DNA binding, either directly or by facilitating appropriate protein–protein contacts, produces additional conformational changes in the receptors so that new serine residues become substrates for protein kinases. This DNA-dependent phosphorylation stage fails in the DBD_{Cys} mutant and the ZK98299-occupied receptors.

The model leads to several predictions and raises several questions:

(*i*) We note that this is a minimal model. Although at least three phosphorylation stages have been demonstrated, more are possible. For example, our data cannot distinguish between a model in which hormone-induced, DNA-independent stage P_2 phosphorylation precedes dimerization (as shown in Fig. 6) or one in which dimerization precedes stage P_2. That hormone binding and dimerization each promote different allosteric changes, leading to a four-stage phosphorylation cascade composed of two hormone-induced, DNA-independent substeps, cannot be ruled out by our data. Dimerization mutants could resolve this question. The role, if any, of accessory proteins, or of phosphatases, is not addressed, and cell-specific as well as intranuclear differences in kinase distribution and activity may complicate the model further.

(*ii*) The model suggests that allosteric changes, plus a series of kinases acting in different cellular compartments, control receptor phosphorylation. The kinases that catalyze phosphorylation of receptor proteins *in vivo* have not yet been identified, and, despite reports to the contrary (43, 44), autophosphorylation of receptors is unlikely (45, 46). PRs have been shown to be excellent substrates for *in vitro* phosphorylation by various phosphoprotein kinases, but usually either inappropriate serine residues or inappropriate amino acids become phosphorylated (10, 47). This could mean either that unsuitable *in vitro* conditions or kinases were used in these experiments or that the receptors were in an improper allosteric configuration for accurate site-specific phosphorylation (see *iii*). We would expect that one or more nuclear protein kinases catalyze the majority of hormone-induced PR phosphorylation. It is now becoming clear that protein kinases in nuclei of eukaryotic cells, some of them

DNA activated (48, 49), exert regulatory control of a number of transcription factors after they bind DNA (25). Since the DBD_{Cys} mutant was intranuclear, an inappropriate cytoplasmic location can be ruled out as a trivial reason for its failure to be fully phosphorylated.

(*iii*) If, indeed, phosphorylation occurs in a stepwise cascade, then we would predict that for accurate *in vitro* and *in vivo* phosphorylation, different kinases will require receptors in different conformational or phosphorylation states generated by an ordered series of reactions. Once one or more phosphates are added, that receptor molecule would become the preferred substrate for the subsequent kinase and phosphorylation stage.

(*iv*) As to the function of hormone-induced phosphorylation, the sites phosphorylated in a DNA-independent manner may relieve the DNA binding repression imparted by accessory proteins, such as heat shock proteins, and/or promote dimerization. We predict that if the serine residues phosphorylated in a DNA-independent manner are mutated to aspartate or glutamate, which mimic the negative charge of the phosphate group, a receptor would be generated that binds DNA constitutively in the absence of hormone. Phosphorylation of such sites may explain recent demonstrations that dopamine activates an orphan of the steroid receptor superfamily for which no ligand has been identified (50) or that cAMP-dependent protein kinase activates chicken PR in the absence of progesterone (51).

Finally, phosphorylation of the DNA-dependent sites may lead to a transcriptionally active receptor, as is the case for other transcription factors (24–26, 49). Mutation of these sites could generate a transcriptionally inactive receptor with otherwise normal hormone and DNA binding properties. Since phosphorylation intensity (ref. 9; Fig. 5), DNA occupancy time (7), and allosteric conformation (42) all differ when hPRs are bound by type II progestin antagonists compared to agonists, could altered phosphorylation at these DNA-dependent sites by type II antagonists explain their inhibition of transcription?

Note Added in Proof. Recent studies showing that ZK98299-occupied receptors fail to dimerize suggest that the DNA-independent P2 phosphorylation precedes dimerization.

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