Role of Phosphorylation on DNA Binding and Transcriptional Functions of Human Progesterone Receptors*

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To study the function of human progesterone receptor (hPR) phosphorylation, we have tested four sets of serine to alanine substitution mutants: 10 serine clusters, located in regions common to both hPR isoforms (the M-series mutants) were mutated in A-receptors and B-receptors; 6 serine clusters located in the B-upstream segment (BUS; the B-series mutants) were mutated individually and collectively and cloned into B-receptors and into BUS-DBD-NLS, a constitutive transactivator, in which the AF3 function of BUS is fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of hPR. Transcription by most of the M-series mutants resembles that of wild-type A- or B-receptors. Mutation of 3 sites, Ser190 at the N terminus of A-receptor mutants, resembling that of wild-type A- or B-receptors. Mutation of 3 sites, Ser190 at the N terminus of A-receptors, a cluster of serines just upstream of the DBD, or Ser195 in the hinge region, inhibits transcription by 20–50% depending on cell or promoter context. These sites lie outside the AF1 activation function. M-series mutants are substrates for a hormone-dependent phosphorylation step, and they all bind well to DNA. Progressive mutation of the B-series clusters leads to the gradual dephosphorylation of BUS, but only the 6-site mutant, involving 10 serine residues, is completely dephosphorylated. These data suggest that in BUS alternative serines are phosphorylated or dephosphorylated at any time. However, even when BUS is completely dephosphorylated, both BUS-DBD-NLS and full-length B-receptors remain strong transactivators. Mutant B-receptors also do not acquire the dominant negative properties of A-receptors, and they retain the ability to activate transcription in synergy with 8-Br-cAMP and antiprogesterins. We conclude that phosphorylation has subtle effects on the complex transcriptional repertoire that distinguishes the two hPR isoforms and does not influence transactivation mediated by AF1 or AF3, but subserves other functions.

The steroid/thyroid receptor family of proteins are ligand-activated transcription factors. Like many other transcription factors, steroid receptors are phosphorylated at sites and for functions that are under intensive study (1–4). The phosphorylation sites of steroid receptors, including chicken and human progesterone receptors (PR), generally (5–10) but not always (11–14) map to serine residues in the N terminus upstream of the DNA binding domain (DBD). One site in cPR maps to the hinge region immediately downstream of the DBD.

Four phosphoserines have been sequenced in cPR; all in Ser-Pro proline-directed kinase consensus sites. Of 5 confirmed sites in hPR, only 1, Ser345, shares homology with a known cPR site (Ser245). Three of the sequenced hPR sites are in BUS, the 164-amino acid B-upstream segment unique to the B-isoform (15, 16). These are Ser81, Ser102, and Ser162, both Ser-Pro proline-directed kinase motifs. The 2 other confirmed hPR sites, Ser294 and Ser345 in the N terminus, are also Ser-Pro suggesting that kinases involved are highly conserved (17–19).

Serine/threonine kinases, including cAMP-dependent protein kinase, mitogen-activated protein kinase, a polypeptide-dependent kinase, CKII, cyclin-dependent kinase (CdK)2, and double-stranded DNA-dependent kinase, all phosphorylate purified cPR or hPR in vitro (15, 16, 20–22). Few sites have been sequenced, however, with the exception of Ser81 of hPR which is correctly phosphorylated by CKII in vitro and Ser528 of cPR which is phosphorylated by cAMP-dependent protein kinase in vitro and lies in close proximity to, but is not identical with, the authentic in vivo phosphorylated hinge region Ser530.

It remains unclear whether studies showing cross-talk between cell surface signaling pathways and nuclear steroid receptors (11, 23–26) are related to PR phosphorylation. In vivo treatments that raise cellular cAMP levels increase cPR-mediated transcription in a ligand-independent manner, but have not been shown to increase phosphate incorporation by the receptors (27–29). Transactivation by hPR is also increased by treatments that raise cellular cAMP levels; an effect that requires ligand occupancy (30, 31). However, the robust transcription produced by synergism between cAMP-dependent protein kinase and ligand-occupied hPR is not accompanied by obvious changes in the phosphorylation state of the receptors. Of the 5 sequenced phosphoserines in hPR and 4 in cPR, none have been shown to be phosphorylated by cAMP-dependent protein kinase or protein kinase C.

The abbreviations used are: PR, progesterone receptor; cPR, chicken PR; hPR, human PR; DBD, DNA binding domain; HBD, hormone binding domain; BUS, B-upstream segment; NLS, nuclear localization signal; CK, casein kinase; ER, estrogen receptor; GR, glucocorticoid receptor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; MMTV, murine mammary tumor virus; tk, thymidine kinase; HSV, herpes simplex virus; PRE, progesterone response element; ERE, estrogen response element; mAb, monoclonal antibody; TAT, tyrosine aminotransferase; Ad2MLP, adenovirus 2 major late promoter.

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There are two phosphorylation states of hPR: a basal state characteristic of unliganded holoreceptors and a ligand-induced state in which phosphate incorporation is severalfold higher than basal. The ligand-induced hyperphosphorylation of hPR is further subdivided into a DNA binding-independent stage and a DNA binding-dependent stage (32, 33). However, the function of phosphorylation remains unknown. It has been speculated to play a role in regulation of transcription and, indeed, for human estrogen receptors (hER) and mouse glucocorticoid receptors (mGR), modest reductions in transcriptional activity have been observed using site-directed mutants. Bai et al. (37) have reported that phosphorylation of Ser530 in the hinge region of cPR increases their transcriptional activity; an effect observed only at low ligand concentrations. On the other hand, mutation of all 5 putative phosphoserines in tau1 of hGHR has no effect on transcription (34). Of course, a role for phosphorylation in functions other than transcription is also possible, as, for example, in ligand-induced dimerization or DNA binding (35, 36).

Analysis of phosphorylation function in hPR is complicated by the existence of two isoforms: B-receptors which contain BUS at their N termini and A-receptors which lack it. B- and A-receptors have important functional differences in response to agonists (38–40) and differ extensively when occupied by antagonists (31, 41, 42). These differences appear to reside in BUS, which contains a strong autonomous activation function (AF3) and is heavily phosphorylated (6, 15, 40). No phosphoserines have yet been localized within AF1, which lies in the N terminus just upstream of the DBD. Two phosphoserines map to the region between the A-receptor translation start site and AF1 which includes a region that possesses a transcriptional inhibitory function in the context of A-receptors.

We have undertaken an extensive series of studies to test the role of hPR phosphorylation on DNA binding and transcriptional activity and constructed two series of serine to alanine substitution mutants. The M-series mutants (Fig. 1) involve 10 clusters of serine residues located in the N-terminal arm or hinge region common to both isoforms, cloned into the background of either A- or B-receptors, and include all Ser-Pro and potential CKII motifs in or around AF1. The B-series mutants (Fig. 1) involve 6 serine clusters located in BUS, cloned into BUS-RBD-NLS (40) and into full-length B-receptors, and include all Ser-Pro motifs that might influence AF3. Using the M-series mutants in the background of full-length B- or A-receptors, we observe no effects on DNA binding with any of the mutants and modest effects on transactivation, dependent on cell and promoter context with 3 out of 10 mutants. Using the B-series mutants, we find that completely dephosphorylated BUS-RBD-NLS constructs retain the strong AF3 transactivating capacity of their wild-type counterparts. Additionally, the unique properties of RU486-occupied full-length B-receptors are retained despite complete BUS dephosphorylation. We conclude that phosphorylation has subtle over-all effects on hPR transcription and that neither the activation function of AF3 in BUS, nor of AF1 in the N terminus, is controlled by its phosphorylation state.

MATERIALS AND METHODS

Plasmid Constructions—Complementary DNAs, hPR2 and hPR1, encoding A- and B-receptors, respectively, digested into the pS3G expression vector (44) were gifts from P. Chambon (Strasbourg, France). BUS-RBD-NLS cloned into pSG5 was described in Sartorius et al. (40). M- and B-series site-specific serine to alanine substitution mutants were made either by oligonucleotide-directed mutagenesis employing a single-stranded template DNA (45) or by polymerase chain reaction (PCR) using overlapping primer products to generate a heteroduplex with the mutant residues placed within a DNA fragment containing convenient restriction sites at the 5′ and 3′ ends (46). For screening purposes, new restriction sites were introduced within or adjacent to the nucleotide sequence associated with the serine to alanine mutation. Individual mutants, particularly those within the B-series BUS-RBD-NLS background, were grouped to form combination mutants. The B2 mutant was constructed by PCR amplification of a fragment in the B2 mutant containing AvrI and SacI restriction sites at the 5′ and 3′ ends, respectively. This fragment was then subcloned into the large AvrI/SacI vector-containing fragment of the B2 mutant plasmid. Ligation at the SacI site recreated the B2 mutant resulting in the B12 combination mutant, which was confirmed with an AvrI/PstI fragment in the B12 mutant plasmid. This fragment was then subcloned into the AvrI/PstI-digested B12 mutant plasmid. The B123 combination mutant was constructed by PCR amplification of a PstI/BglII fragment from the B12 mutant plasmid and was subcloned into the PstI/BglII-digested B123 mutant plasmid. The B1234 combination mutant was constructed by PCR amplification of a PstI/BglII fragment from the B123 combination mutant plasmid, which was subcloned into the B123 combination mutant plasmid. The B4s combination mutant plasmid was constructed by digesting the B1 and B2 plasmids with BstII/RsaI and subcloning the small fragment from the B1 plasmid into the large fragment from the B2 plasmid. The CK mutant was constructed in both the wild-type BUS-RBD-NLS construct and the BUS-RBD-NLS combination mutant, and individual and combination mutants were inserted into the hPR1 plasmid encoding full-length B-receptors by subcloning either an EcoRI/BstII or EcoRI/RsaI fragment from the mutant BUS-RBD-NLS plasmid into the hPR1 wild-type plasmid. All mutants were verified by deoxynucleotide sequencing. All mutant plasmids were transfected into COS-1 cells, HeLa cells, and the molecular size and structure of the expressed proteins were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting as described previously (32).

Cell Transfections—Transient transfections into PR-negative COS-1 monkey kidney epithelial, HeLa human cervicocarcinoma, and T47D human breast cancer cells were performed by calcium phosphate precipitation as described previously (31). Receptors included wild-type and mutant hPR expression plasmids and the human estrogen receptor (hER) expression vector HEGO (47) (a gift of P. Chambon). Reporter plasmids PRE- tk, PRE- TATA, and MMTV-CAT were gifts of P. Chambon. PRE- TATA- cat was constructed as described previously (31), and the two PREs were replaced by two EREs derived from the estrogen responsive promoter to generate PRE- TATA- cat- ERE. Nuclear-cytosol expression plasmids, pCH110 (Pharmacia Biotech Inc.) or CMV- β-gal (Clontech, Palo Alto, CA), were used to correct for transfection efficiency, and the Bluescribe plasmid (Stratagene) was used as a carrier. Wild-type and mutant expression plasmids were also transiently transfected into HeLa cells containing the stably integrated MMTV-CAT reporter construct introduced into these PR-negative cells as described previously (48).

[32P]Orthophosphate Labeling, Immunoprecipitation, and Immunoblotting—COS-1 cells transiently transfected with full-length hPR1, hPR2, or their respective mutants, were treated with R5020 or alcohol vehicle 10 min prior to the addition of [32P]orthophosphate (0.15 μCi/ml of medium) as described previously (6). Cells were harvested 4 to 17 h after incubation with [32P]orthophosphate, homogenized in buffer containing 0.6 M KCl, desalted over Sephadex G-25, and immunoprecipitated with B-30 and/or AB25 monocular antibodies. Immunoprecipitated receptors were then subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with mouse B-30 and or AB52, and the signals were visualized using x-ray film by autoradiography of luminescence (ECL, Amersham), as described previously (48). The sheets were air-dried, the chemiluminescence was allowed to decay over 24 h, and the 32P radioactivity present in receptor-associated bands was visualized by autoradiography of another x-ray film.

Gel Shift Assays—Gel mobility shift assays were performed as described (31) using whole-cell extracts prepared from COS-1 cells. Hormone (0.1 μM R5020) was added 2 h prior to cell harvest. 32P-Labeled oligonucleotide probes were 27 base pairs in length and contained either a palindromic progesterone response element (PRE) from the tyrosine aminotransferase (TAT) promoter, or the distal palindromic PRE of the MMTV long terminal repeat (30, 32).

CAT Assays—Twenty-four h after transfection, the cell medium was changed and the cells were incubated with or without R5020 for an additional 24 h. Cells were then harvested, and lysates were analyzed for chloramphenicol acetyltransferase (CAT) activity by thin layer chro.
The specific amino acids mutated, and their designations are illustrated in Fig. 1. Among the mutated residues are ones that have been identified as phosphoserines in hPR (α in Fig. 1) including Ser102 (B3), Ser162 (B5), and Ser345 (M3); ones that have been identified as phosphoserines in cPR and bear homology to sites in hPR including Ser345 (M3) and Ser676 (MH); and ones that represent consensus phosphorylation sites for CKII and proline-directed kinases and have at least a Ser-Pro motif. In the M-series mutants, 10 clusters of serine residues located downstream of Met165 in regions common to both PR isoforms were mutated in expression vectors encoding both isoforms. In the B-series mutants, 6 clusters of serine residues located in BUS were mutated in BUS-DDB-NLS and in the full-length B-receptors. Additional B-series mutants contained two or more of the mutant clusters in various combinations, and, in the CK (1-5) construct, all 6 serine clusters in BUS were mutated simultaneously.

M-Series Mutants: Phosphorylation Sites Common to Both A- and B-Receptors—Initial studies involved a series of 9 different serine to alanine mutant clusters located in the N terminus of A-receptors upstream of the DBD (designated M1 to M9) and 1 located in the hinge region downstream of the DBD (designated MH) (Fig. 1). Mutants M5 to M9 either surround or are located within the AF1 transcription activation domain. The mutant proteins were all well expressed as demonstrated by immunoblotting (not all shown, but see Fig. 3). They were tested for transcriptional activity by transient cotransfection with the minimal PRE2-TATAAD2MLP and PRE2-TATANk promoters or the complex PRE-tkHSV promoter, using either HeLa or COS cells. Transcription of the CAT gene by a majority of these constructs when occupied by R5020 was no different than transcription by wild-type A-receptor controls (data not shown). Three sets of A-receptor mutants, M1, M9, and MH, described in Fig. 1, have some inhibitory effects on transcription, that are promoter- and cell-specific (Figs. 2, A and B). In COS cells (Fig. 2A), M1 mutants were less active on the simple PRE2-TATAAD2MLP promoter than on the complex PRE-tkHSV promoter; M9 and MH were weaker receptors than wild-type A-receptors on both promoters. In HeLa cells (Fig. 2B), transcription controlled by M1 and M9 is variable and promoter-dependent, and no clear rules can be deduced. The MH hinge region mutation at Ser676 appears to have the most consistent deleterious effect on A-receptor activity. The data in Fig. 2 represent average values for duplicates of 2–8 assays per set and include the range of variability among assays. The overall impression is that these three mutants have a 20–50% transcription inhibitory effect. Mutants M1, M9, and MH were also cloned into the B-receptor expression vector and tested on PRE2-TATAAd2MLP in HeLa cells, with results analogous to those seen with their A-receptor counterparts (data not shown).

We conclude that phosphorylation of Ser102, the M9 cluster, or Ser676 has subtle effects on hPR transcription. Bai et al. (37) have reported that mutation of Ser530 in cPR (which is homologous to mutant MH at Ser676 in hPR) reduces receptor-mediated transcription in transient transfections assays, but only at low hormone concentrations. We observe a transcriptional decrement even at saturating hormone concentrations with MH. Since there is no evidence that Ser676 is phosphorylated in hPR, it is possible that the decrement in transcriptional activity observed with MH is due to disruption of a function of this domain independent of a phosphorylation event (49). Our results with hPR are analogous to those obtained with hER, in which site-directed N-terminal (AF1) mutants (24, 50) also produced modest cell- and promoter-specific reductions in transcriptional activity in transient transfection assays. Similarly, mutation of all 7 phosphorylated residues in the N terminus of hGR, 6 of which lie within tau1, reduced transcription by 30–40% in transient assays (51). However, when this 7-site mGR mutant was expressed at physiological levels, transcription by the mutant was equivalent to the wild-type mGR. These findings illustrate the complexities involved in assessing subtle functional effects using overexpressed mutant receptors in transient transfection assays.

The three M-series mutants of interest were also analyzed for their ability to undergo phosphorylation-dependent structural changes (Fig. 3A) and for their ability to bind DNA at a PRE (Fig. 3B). For these studies, wild-type A-receptors and all the M-series mutants including the M1, M9, and MH mutants were expressed in COS cells, treated with R5020, or left untreated, and the extracted receptors were analyzed by immunoblotting and gel mobility shift assays. As we have shown previously (17), unactivated wild-type A-receptors immunoblotted as singlets (Fig. 3A, solid arrow), but, after activation by hormone, they migrate as doublets on electrophoretic gels (Fig. 3A, open arrow) due to a hormone-dependent phosphorylation step. Analogous to wild-type A-receptors, M1, M9, and MH are also singlets in the absence of hormone and are upshifted by hor-
We conclude that the serines mutated in these three constructs are not targets for the hormone-dependent phosphorylation that produces the upshift. Mutation of 6 otherserineclustersintheNterminusofA-receptors(seeFig.1)alsohadnoeffectontheirimmunoblottingpattern(datanotshown). On the other hand, the M3 mutant, which includes Ser345, is upshift-deficient (data not shown) consistent with recent reports of Zhang et al. (15). Thus, the hormone-dependent upshift appears to be unrelated to transcriptional activity, since a mutant lacking the upshift (M3) is fully active, while mutants with a normal upshift (M1, M9, MH) are transcriptionally deficient.

Other recent studies (13, 35, 52) have suggested that phosphorylation of steroid receptors regulates their DNA binding capacity. To test this, wild-type A-receptors and the M1, M9, and MH mutants were expressed in COS cells in the presence or absence of a saturating concentration of R5020. The receptors were extracted and incubated with a 27-base-pair 32P-labeled oligonucleotide containing either the distal palindromic PRE of the MMTV long terminal repeat (not shown) or a palindromic PRE from the TAT promoter (Fig. 3). Receptor-DNA complexes at three different extract concentrations were then analyzed by the in vitro gel mobility shift assay. Fig. 3 shows that there is no remarkable difference in DNA binding affinity between wild-type and mutant A-receptors. Similar conclusions were drawn from a study comparing wild-type B-receptors and their corresponding M1, M9, and MH mutants (data not shown). It is unlikely, therefore, that altered DNA binding activity or differences in protein expression levels account for the reductions in transcription seen with the M1, M9, and MH mutants, since the PRE used in the gel mobility shift assay was also inserted into all the reporter plasmids, and comparable levels of wild-type and mutant receptors were expressed from transiently transfected COS cells (see Fig. 3A).

Phosphorylation Sites Unique to B-Receptors: the B-Series Mutants—We have previously shown that PR B- and A-receptors have important functional differences due to an AF3 present in BUS (40). BUS is also highly phosphorylated (6). The triplet immunoblotting banding pattern of full-length 120-kDa BUS, which is due to phosphorylation, is entirely reproduced by the 20-kDa BUS fragment (see Fig. 4). Because of its strong transactivating capacity and intensive phosphorylation, BUS-DBD-NLS is an ideal receptor fragment with which to test the functions of phosphorylation. We therefore constructed a set of BUS phosphorylation mutants in which 6 clusters of serine residues were individually or collectively mutated. Five of these clusters (B1 to B5) contain Ser-Pro phosphorylation motifs; the sixth (Bck) has a CKII phosphorylation motif. B1 is mutated at Ser20, B2 at Ser25, B3 at Ser99,100,101,102, B4 at Ser131, B5 at Ser162, and Bck at Ser79,81 (Fig. 1). Three series
in these constructs, Ser\textsuperscript{102} in B\textsubscript{3}, Ser\textsuperscript{162} in B\textsubscript{5}, and Ser\textsuperscript{81} in B\textsubscript{CK} have been sequenced (\(\ast\) in Fig. 1) and are known to be phosphoserines (15, 16). Additionally, B\textsubscript{2} was combined with B\textsubscript{5} to yield B\textsubscript{12}, and, similarly, B\textsubscript{123}, B\textsubscript{1234}, and B\textsubscript{12345} were constructed. Finally, all 6 clusters were simultaneously mutated in a construct called B\textsubscript{CK(1–5)} (Fig. 1). The BUS mutants were inserted into BUS-DBD-NLS and into full-length B-receptors.

Fig. 4 shows immunoblots that demonstrate structural features of some of these mutants, which are well expressed suggesting that their stability is not altered by the BUS mutations. Full-length B-receptors transiently expressed in COS cells resolve as triplets on electrophoretic gels (Fig. 4A) and resemble natural B-receptors isolated from breast cancer cells. Addition of hormone has minor effects on the banding pattern (compare lanes 1 and 2) since the hormone-dependent M\textsubscript{1} upshift observed in other cells is less prominent in COS cells (see Fig. 4C). Mutation of any one Ser-Pro motif, as in the B\textsubscript{3} (Fig. 4A, lanes 3 and 4) or the B\textsubscript{CK} (lanes 7 and 8) mutants does not alter the immunoblotting pattern. These two represent serine residues that are known to be phosphorylated in vivo. However, the multiple banding pattern is reduced to a singlet if 5 (lane 5) or all 6 (lane 9) serine clusters are mutated. Nevertheless, at least 1 hormone-dependent phosphorylation site is retained in these constructs (lanes 6 and 10), since the M\textsubscript{1} upshift occurs after R5020 treatment (compare lanes 9 and 10, for example) confirming that this site(s) lies downstream of BUS in the region common to both PR isoforms (15).

BUS-DBD-NLS also immunoblots as a triplet (Fig. 4B, lane 1) due to phosphorylation of sites located in BUS (lane 3) as we have shown previously (40). The complexity of this pattern, coupled with high performance liquid chromatography analysis of tryptic phosphopeptides (6, 16) suggest that it is due to phosphorylation of multiple serine residues. If BUS-DBD-NLS is treated with calf intestinal alkaline phosphatase, the higher M\textsubscript{1} hyperphosphorylated bands can be reduced or eliminated (Fig. 4B, lane 2). This is also demonstrated using the BUS\textsubscript{CK(1–5)}-DBD-NLS mutant in which the upper two bands are extensively reduced (lane 5), compared to the wild-type construct which immunoblots as three or more bands (Fig. 4B, lane 4). Complete reduction of the triplet to a singlet is seen in full-length B-receptors in which all 6 serine phosphorylation motifs present in BUS (B\textsubscript{CK(1–5)}) have been mutated (Fig. 5B, compare lanes 6 and 7). We tentatively conclude that CK(1–5) mutants are entirely dephosphorylated at the sites unique to B-receptors.

Immunoblots analyses of wild-type BUS-DBD-NLS and its mutants carrying single or intermediate numbers of serine substitutions are also informative about generation of the triplet structure (Fig. 4C). Regardless of the site involved, mutation of any 1 of the 6 serine clusters, produces no discernible change in the immunoblot banding pattern (data not shown, but see Fig. 4A). As shown in COS cells, even mutation of 2 of the 6 clusters (B\textsubscript{12}) produces no diminution in the number of blotted bands (Fig. 4C, lane 2). Only after 3 (B\textsubscript{123}) or more
Fig. 5. Analysis of the phosphorylation state of B-series mutants based on [\(^{32}\)P]orthophosphate incorporation in intact cells. COS cells expressing the indicated BUS-DBD-NLS or full-length hPRb constructs were incubated with [\(^{32}\)P]orthophosphate for 4 h, nuclear extracts were immunoprecipitated with mAb B-30 alone or together with AB-52, separated by SDS-PAGE, transferred to nitrocellulose, and the sheet was visualized by enhanced chemiluminescence (left panels), then dried overnight and exposed to another x-ray film to generate the autoradiogram (right panels). A, comparison of the protein structure (left panel) and [\(^{32}\)P]orthophosphate incorporation (right panel) of wild-type BUS-DBD-NLS or the corresponding 6-site CK(1–5) BUS mutant. B, comparison of the protein structure (left panel) and [\(^{32}\)P]orthophosphate incorporation (right panel) of full-length B-receptors or the corresponding 6-site CK(1–5) BUS mutant.

(B\(_{1234}\) and B\(_{12345}\)) clusters are mutated does the pattern begin to converge to a single band (lanes 3–5). However, even mutation of 5 of the 6 sites, as for example in mutant B\(_{12345}\) (Fig. 4C, lane 5), still yields a weak doublet (open arrow). These data suggest that there is considerable intramolecular heterogeneity among the sites that are phosphorylated in vivo and that phosphorylation at several alternative combinations of sites can produce the complex triplet banding pattern, as has been described for vitamin D receptors (53).

Also shown in Fig. 4C, lanes 6–11, is a comparison of the immunoblotting pattern of three BUS-DBD-NLS mutants when they are expressed in HeLa cells or COS cells. It demonstrates subtle differences in the phosphorylation pattern produced by the two cell lines that may reflect differences in cellular kinases, differences in the residues that are their targets, or possibly differences in protein expression levels which are usually lower in HeLa cells.

The studies shown in Fig. 5 demonstrate directly that the 6-site BUS mutant, CK(1–5), is completely dephosphorylated. COS cells transiently expressing wild-type BUS-DBD-NLS or the BUS\(_{5(1–5)}\)-DBD-NLS mutant were incubated with [\(^{32}\)P]orthophosphate. The labeled receptors were then extracted, immunoprecipitated, resolved by gel electrophoresis, transferred to nitrocellulose, and analyzed by both [\(^{32}\)P] autoradiography (right panels) and by immunoblotting with mAb B-30 (left panels). Hormone treatment was unnecessary, since the constructs lack an HBD and are constitutive transactivators (40). The immunoblot in Fig. 5A shows the characteristic multiple banding pattern of wild-type BUS-DBD-NLS (lanes 1 and 3) and its reduction to a single band in the BUS\(_{5(1–5)}\) mutant (lanes 2 and 4). The parallel autoradiogram shows that all the protein bands are phosphorylated in wild-type BUS-DBD-NLS (lanes 5 and 7), but that in BUS\(_{5(1–5)}\)-DBD-NLS, even the heavy singlet protein band (lanes 2 and 4) is dephosphorylated (lanes 6 and 8). This confirms that in the 6-site mutant no residues remain that are substrates for endogenous serine kinases, and that no other amino acid residues become alternatively phosphorylated when the fully mutated BUS\(_{5(1–5)}\)-DBD-NLS construct is expressed.

Fig. 5B is a similar analysis of COS cells transiently expressing full-length B-receptors that contain either wild-type BUS or the 6-site B\(_{5(1–5)}\) mutant BUS. Because these receptors have an HBD, the cells were either untreated (–) or treated with R5020 (+) before the receptors were extracted. In the absence of hormone, the characteristic triplet immunoblot banding pattern is observed with wild-type B-receptors (lane 1) and reduced to a singlet in the mutant (lane 2). After hormone occupancy, a slightly shifted banding pattern is observed in the immunoblot of wild-type B-receptors (lane 3), characteristic of COS cells (see Fig. 4). The B\(_{5(1–5)}\) mutant (lane 4) also shifts from a singlet (lane 2, solid arrow) to a doublet (lane 4, open arrow) following hormone occupancy, due to phosphorylation of 1 or more sites downstream of BUS. The parallel [\(^{32}\)P] autoradiogram shows that the basal phosphorylation of wild-type B-receptors (lane 5) is augmented by hormone treatment (lane 7), as we have previously reported (6). That this hormone-dependent hyperphosphorylation is not due to sites in BUS is shown by the B\(_{5(1–5)}\) mutant in which a 4-fold increase in [\(^{32}\)P]orthophosphate incorporation is observed following hormone treatment (lane 8) compared to the untreated control (lane 6).

BUS-DBD-NLS serves as a powerful tool to study functions of phosphorylation because of its strong constitutive transactivation capacity. We have therefore extensively analyzed the DNA binding properties and transcription regulatory properties of constructs containing either a wild-type or a phosphorylation-deficient BUS. We have previously reported (40) that wild-type BUS-DBD-NLS binds strongly to DNA at a PRE if a nuclear accessory protein, or the bivalent mAb B-30, is included in the DNA-bound complex. We find an identical DNA binding pattern with the B\(_{5(1–5)}\) mutant (data not shown). Thus, elimination of BUS phosphorylation does not influence the DNA binding capacity of the BUS-DBD-NLS construct or its ability to interact with the nuclear accessory protein.

We have also carried out extensive transcription analyses comparing fully phosphorylated wild-type BUS-DBD-NLS and full-length B-receptors, with their counterparts containing single-site and multi-site BUS phosphorylation-deficient mutants.
Phosphorylation and Antagonist Actions in B-Receptors; the B-Series Mutants—There are important quantitative differences between the two PR isoforms when they are occupied by agonists (38–40). However, when the two isoforms are occupied by antagonists, differences between them are profound (31, 41, 42). For example, through cross-talk with the cAMP signaling pathway, B-receptors occupied by the antiprogestin RU486 become strong transcriptional activators under conditions in which RU486-occupied A-receptors inhibit transcription. Since B- and A-receptors differ only by the presence or absence of BUS, we asked whether their phosphorylation state influences the unique properties of B-receptors. In Fig. 7, full-length wild-type B-receptors, or their 6-site B(CKL) counterparts, were transiently transfected into PR-negative T47D breast cancer cells (55) together with an MMTV-CAT reporter, and the cells were untreated or treated with R5020 or RU486, with or without 8-Br-cAMP. Lanes 1–5 show that T47D cells transiently transfected only with MMTV-CAT are unresponsive to any treatments because they lack PR. If wild-type B-receptors are introduced into the cells together with MMTV-CAT (lanes 6–13), there is no CAT synthesis in the absence of hormone (lane 12), but CAT levels are high following R5020 treatment (lane 13). RU486 (lanes 6 and 7) or 8-Br-cAMP (lanes 10 and 11) alone is unable to activate transcription, but when the two are combined (lanes 8 and 9), strong CAT activity is observed. Since this unusual synergism between 8-Br-cAMP and RU486 occurs only with B-receptors, we asked whether it is dependent on the phosphorylation state of BUS. The B(CKL) mutant (lanes 14–21) strongly stimulates transcription when occupied by R5020 (compare lanes 20 and 21); RU486 (lanes 14 and 15) and 8-Br-cAMP (lanes 18 and 19) alone are inactive; and the combination of RU486 plus 8-Br-cAMP (lanes 16 and 17) is strongly active. We conclude that this unique agonist-like effect of RU486-occupied B-receptors in synergy with cAMP is not dependent on the phosphorylation state of BUS, and, that despite its complete dephosphorylation, BUS can still support this property.

Another interesting functional difference between the two hPR isoforms is that, when occupied by RU486, A-receptors but not B-receptors inhibit transcription of an estrogen response element (ERE) regulated promoter activated by estradiol-occupied hER. BUS blocks this repressor effect of A-receptors. We asked, in the study shown in Fig. 8, whether this property would be lost by a dephosphorylated BUS. For this, HeLa cells were transiently transfected with the wild-type ER expression vector HEGO (47) either alone or together with expression vectors for wild-type B- or A-receptors or the B(CKL) receptor mutant. Cells were treated or not with 17β-estradiol (E) and RU486 (RU), and transcription was measured from the ERE2-TATA0-CAT reporter. This promoter lacks a PRE and cannot be influenced by PR directly. As shown in Fig. 8, the ERE2-TATA0-CAT reporter is not transcribed by ER in the absence of estradiol (lane 1) but is strongly transcribed in its presence (lane 2). As expected, in the absence of PR, RU486 (lane 3) has no influence on this ER-activated, ERE-regulated promoter. When wild-type B-receptor expression vectors are co-transfected with ER (lanes 4 and 5), RU486 still has no effect, but with co-transfected wild-type A-receptors (lanes 6 and 7), ER-driven transcription is reduced by more than 90%. Note that this inhibitory effect of A-receptors is DNA binding independent, since the promoter lacks a PRE. Despite mutation of all stably replicating MMTV-CAT template were constructed and analyzed because of the possibility that PR vary in their ability to activate chromosomal versus transiently introduced promoters (54). We postulated that the state of PR phosphorylation might explain these differences, but conclude that they do not.

**Fig. 6.** Transcriptional activity of wild-type full-length B-receptors or BUS-DBD-NLS and their corresponding 6-site phosphorylation-deficient mutants. HeLa cells were transiently transfected with 2 μg of PRE2-TATA0-CAT or MMTV-CAT reporters, 2 μg of β-galactosidase expression vector, and 1–250 ng of the wild-type or mutant receptor expression vectors, as shown. Cell lysates were normalized to β-galactosidase activity, and CAT expression was analyzed by TLC and quantified by phosphorimaging. A, BUS-DBD-NLS constructs and PRE2-TATA0-CAT. B, full-length B-receptor constructs and MMTV-CAT or PRE2-TATA0-CAT.

The constructs all have remarkably similar transcriptional activities. An example of such a study, comparing transcription from the PRE2-TATA0-CAT reporter cotransfected into HeLa cells together with increasing concentrations of expression vectors encoding wild-type BUS-DBD-NLS, or the 6-site mutant BUSCK(1–5)-DBD-NLS, is shown in Fig. 6. There is a constitutive, dose-dependent increase in transcription by wild-type BUS-DBD-NLS, which, at its peak, is equivalent to transcription by full-length B-receptors (40). Surprisingly, transcription by the completely dephosphorylated BUSCK(1–5)-DBD-NLS mutant is essentially identical with that of its fully phosphorylated counterpart (Fig. 6A). Minor effects of dephosphorylation are observed at low DNA input concentrations. In Fig. 6A, for example, transcription following transfection by 10 ng of the cDNA encoding wild-type BUS-DBD-NLS is 23% of the maximum seen at 250 ng, while at 10 ng of the cDNA encoding BUSCK(1–5)-DBD-NLS, transcription is 5% of maximum. However, at higher cDNA concentrations, both constructs produce equivalent amounts of CAT activity, and we conclude that the phosphorylation state of BUS has little or no influence over transcription by AF3 in the context of BUS-DBD-NLS.

Fig. 6B shows CAT transcription in R5020-treated HeLa cells driven from the MMTV or PRE2-TATA0 promoters, under the control of full-length hPRB containing either wild-type BUS or completely dephosphorylated BUSCK(1–5). Clearly, there are no remarkable differences between wild-type hPRB and ones carrying dephosphorylated BUS, regardless of the receptor concentration introduced into the cells. We conclude that the phosphorylation state of BUS has little or no influence over AF3 activity in the context of full-length B-receptors.

Similar conclusions were reached using BUS-DBD-NLS constructs and MMTV-CAT when the reporter was stably transfected into HeLa cells (data not shown). HeLa cells with a
Fig. 7. Antagonist-occupied B-receptors that are phosphorylation-deficient become transactivators when cAMP levels are raised. T47D_B cells were transiently transfected with 1 µg of the MMTV-CAT reporter and 1 µg of the pSG5 expression vector alone (lanes 1–5) or the vector encoding wild-type B-receptors (lanes 6–13), or the 6-site BUS mutant (lanes 14–21). Twenty-four hours after transfection, cells were either untreated (−) or treated with 1 mm 8-Br-cAMP (cAMP), 50 nm R5020 (R), 100 nm RU486 (RU), or the indicated combinations for 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantified by phosphorimaging.

Fig. 8. Full-length B-receptors carrying a completely dephosphorylated BUS do not acquire the inhibitory phenotype of A-receptors. HeLa cells were transiently co-transfected with 2 µg of ERα-TATA_box-CAT and 5 ng of the ER expression vector HEGO, with or without 250 ng of expression vectors for full-length A- or B-receptors, or the 6-site BUS mutant B-receptors. Cells were untreated (−) or treated with 10 nm 17β-estradiol (E) and/or 100 nm RU486 (RU) as shown. Cell extracts normalized to β-galactosidase activity were analyzed for CAT activity as described.

| Receptors | Ligands | RU | cAMP | R | RU | cAMP | - | R | RU | cAMP | - | R | RU | cAMP | - | R |RU | cAMP | - | R |
|-----------|---------|----|------|---|----|------|---|----|---|------|---|----|---|------|---|----|---|------|---|----|
| No Receptor | ER only | | | | | | | | | | | | | | | | | | | |
| hPRa | | | | | | | | | | | | | | | | | | | | |
| hPRback (1-5) | | | | | | | | | | | | | | | | | | | | |

BUS phosphorylation sites in B_back (lanes 8 and 9), this dominant repressor activity of A-receptors cannot be reconstituted in B-receptors. We conclude again, based on a different experimental model, that factors other than the phosphorylation state of BUS control the unique transcriptional properties of full-length B-receptors.

Summary—In summary, we asked whether phosphorylation of hPR regulates their DNA binding and transcriptional properties. We mutated a number of putative or known phosphorylation sites in the N-terminal region (the M-series mutants) common to the A- and B-isotypes. Many of these sites are either within or bordering AF1, but most mutations had no appreciable effects on transcription by either isoform. Two mutants (M1, M9) in the N terminus and one in the hinge region (MH) produced modest decrements in transcription comparable in magnitude to those seen with mutant hER and mGR (24, 50, 51). If these effects are authentic, it would suggest that receptor phosphorylation does not function as an on/off switch, but rather as a fine-tuning mechanism. On the other hand, if phosphorylation of steroid receptors does not affect receptor-activated transcription as has been shown for hGR and rabbit PR (34, 43), it suggests that receptor processes not directly linked to transcription should be explored.

Similarly, through a combination of site-directed serine to alanine mutations in the BUS region of B-receptors (the B-series mutants), we were able to generate a phosphorylation-deficient AF3 activation domain, which in wild-type B-receptors is highly phosphorylated at multiple serine residues. We studied the autonomous activity of AF3 in BUS-BDB-NLS and its cooperativity with AF1 and AF2 in the context of full-length B-receptors in transfection assays utilizing (a) cultured cells derived from different tissues, (b) simple and complex promoters, (c) different levels of protein expression, and (d) templates that are transiently or stably introduced and presumably contain a poorly or a more regularly organized nucleosome structure. Under these extremes of assay conditions, the autonomous transcription efficiency of AF3, as well as its ability to additively or synergistically complement the activities of AF1 and AF2 in the full-length receptors, was essentially unaffected by the mutations that dephosphorylate BUS. Even when we examined functional responses that are specific for B-receptors, such as the agonist activity of antagonist-bound B-receptors in the presence of cAMP, or the inability of B-receptors to be dominant-negative inhibitors of ER, we again found that receptors which were fully phosphorylated or dephosphorylated in BUS acted identically. These B-receptor-specific responses have an absolute requirement for BUS and presumably are mediated by conformational changes in BUS that lead to altered intra- or intermolecular interactions. It is therefore surprising that the intense phosphorylation seen on the BUS fragment is not involved in these activities, but we can come to no other conclusion.

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