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Glucocorticoid Receptor Phosphorylation Differentially Affects Target Gene Expression

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The glucocorticoid receptor (GR) is phosphorylated at multiple sites within its N terminus (S203, S211, S226), yet the role of phosphorylation in receptor function is not understood. Using a range of agonists and GR phosphorylation site-specific antibodies, we demonstrated that GR transcriptional activation is greatest when the relative phosphorylation of S211 exceeds that of S226. Consistent with this finding, a replacement of S226 with an alanine enhances GR transcriptional response. Using a battery of compounds that perturb different signaling pathways, we found that BAPTA-AM, a chelator of intracellular divalent cations, and curcumin, a natural product with antiinflammatory properties, reduced hormone-dependent phosphorylation at S211. This change in GR phosphorylation was associated with its decreased nuclear retention and transcriptional activation. Molecular modeling suggests that GR S211 phosphorylation promotes a conformational change, which exposes a novel surface potentially facilitating cofactor interaction. Indeed, S211 phosphorylation enhances GR interaction with MED14 (vitamin D receptor interacting protein 150). Interestingly, in U2OS cells expressing a nonphosphorylated GR mutant S211A, the expression of IGF-binding protein 1 and interferon regulatory factor 8, both MED14-dependent GR target genes, was reduced relative to cells expressing wild-type receptor across a broad range of hormone concentrations. In contrast, the induction of glucocorticoid-induced leucine zipper, a MED14-independent GR target, was similar in S211A- and wild-type GR-expressing cells at high hormone levels, but was reduced in S211A cells at low hormone concentrations, suggesting a link between GR phosphorylation, MED14 involvement, and receptor occupancy. Phosphorylation also affected the magnitude of repression by GR in a gene-selective manner. Thus, GR phosphorylation at S211 and S226 determines GR transcriptional response by modifying cofactor interaction. Furthermore, the effect of GR S211 phosphorylation is gene specific and, in some cases, dependent upon the amount of activated receptor. (*Molecular Endocrinology* 22: 1754–1766, 2008)

THE GLUCOCORTICOID RECEPTOR (GR) is a transcription factor responsible for managing developmental and metabolic processes in response to glucocorticoids (1–4). Before ligand binding, the GR is predominantly cytoplasmic and is bound to the heat shock protein 90-p23 chaperone complex (5–7), which is thought to inactivate GR DNA binding and transcriptional regulatory functions, while maintaining the receptor competent for a high-affinity ligand binding (8, 9). Upon ligand binding, the hormone-receptor complex translocates to the nucleus where it binds specific DNA sequences and modulates transcription. Although GR is expressed in virtually every mammalian cell type, it regulates the expression of distinct sets of genes in a promoter- and cell type-specific manner (3, 10). Even though the determinants of this promoter selectivity are not completely understood, our recent findings suggest that components of the Mediator complex, such as MED14, associate with GR and contribute to gene-specific regulation (11).

Like many other regulators, GR is a phosphoprotein (12, 13). The ligand-free receptor is phosphorylated, with additional phosphorylation events occurring in conjunction with ligand binding (14). GR isolated from cultured mammalian cells or ectopically expressed in yeast is phosphorylated on multiple sites (15). Three sites cluster to the N-terminal transcriptional regula-

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Abbreviations: AF-1, Activation function 1; AR, androgen receptor; Cdk2, cyclin-dependent kinase 2; Dex, dexamethasone; ER, estrogen receptor; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HA, hemagglutinin; IGFBP1, IGF-binding protein 1; IRF8, interferon regulatory factor 8; JNK, c-Jun N-terminal kinase; LAD1, ladinin 1; LXR, liver X receptor; MMTV, mouse mammary tumor virus; PSA, prostate-specific antigen; RT, room temperature; TBS, Tris-buffered saline; WT, wild type.

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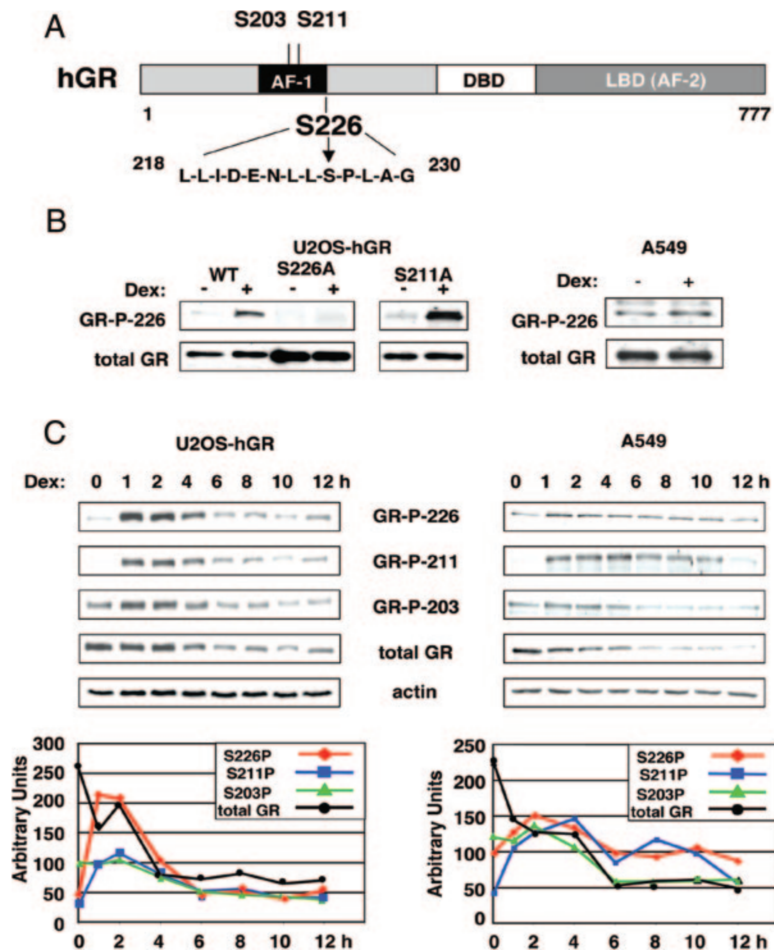

Fig. 1. GR Structure, Specificity, and Kinetics of S226 Phosphorylation

A, Functional domains and phosphorylated residues of the hGR. Shown is a schematic representation of hGR with major phosphorylation sites and the sequence of the hGR phosphopeptide used to produce the phospho-S226-specific antibody. B, Immunoblotting of hGR with phospho-S226 antibody is shown. Whole-cell extracts prepared from U2OS cells expressing an HA-tagged hGR (U2OS-hGR), either WT or phosphorylation site mutants S226A or S211A, or A549 cells expressing endogenous GR, untreated or treated with 100 nM Dex for 1 h, were analyzed by immunoblotting with phospho-S226 (*top panel*), or a phosphorylation state-independent GR antibody (*bottom panel*) as a measure of total GR. C, Kinetics of S226, S211, and S203 phosphorylation in response to Dex treatment. U2OS-hGR or A549 cells were treated with ethanol (−) or Dex (100 nM) for the time indicated. Whole-cell lysates were prepared, normalized for total protein concentration, and analyzed by immunoblotting with phospho-S226, phospho-S203, phospho-S211, phosphorylation state-independent GR (total GR), and actin antibodies. Quantitative analysis of immunoblot results in panel C normalized to actin. DBD. DNA-binding domain; LBD, ligand-binding domain.

tory domain and include serine 203 (S203), serine 211 (S211), and serine 226 (S226) in the human (h) GR numbering scheme (Fig. 1A) (16). We have demonstrated previously that cyclin E/cyclin-dependent kinase 2 (Cdk2) phosphorylates GR at S203, whereas cyclin A/Cdk2 phosphorylates both S203 and S211 (15). Mutations at these sites, or of particular Cdk genes in yeast, reduce GR-dependent transcriptional activation, suggesting that phosphorylation of S203 and S211 is required for full GR transcriptional enhancement. Furthermore, mammalian cells lacking a Cdk inhibitor $p27^{KIP1}$ display a concomitant increase in cyclin/Cdk2 activity and GR phosphorylation at the Cdk sites, and enhanced receptor transcriptional activity (17). In addition, GR S211 also appears to be a substrate for p38 MAPK (18). In contrast, phosphorylation of S226 by c-Jun N-terminal kinase (JNK), another member of the MAPK family, inhibits GR transcriptional activation (19). Phosphorylation of S226 by JNK has also been shown to regulate GR export from the nucleus upon hormone withdrawal (20).

GR dephosphorylation at S203 and S226 is regulated by protein phosphatase 5 (21), whereas S211 phosphatase is as yet unidentified. The fact that protein phosphatase 5 differentially affects GR target gene expression further suggests a link between GR phosphorylation and transcriptional regulation. We have recently shown that GR phospho-isoforms selectively occupy receptor target genes (22). Yet mech-

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anistically how GR phosphorylation regulates gene expression remains enigmatic.

In this study we demonstrate gene-specific differences in the requirement for GR phosphorylation. Our data further show that the phosphorylation state of S211 is a key regulator of receptor transcriptional activation and repression.

RESULTS

Characterization of hGR S226 Phosphorylation Site-Specific Antibody

The polyclonal antibody GR phospho-S226 was raised against the phosphopeptide LLIDENLLS(P)PLAG corresponding to residues 218–230 of the hGR (Fig. 1A). The antibodies were tested for their ability to detect GR by immunoblotting extracts from U2OS cells stably expressing an hemagglutinin (HA)-tagged hGR (U2OS-hGR) either untreated or treated for 1 h with dexamethasone (Dex). The phospho-S226 antibody showed substantial immunoreactivity toward GR from Dex-treated, but not untreated, cells, even though equal amounts of GR were present as determined by immunoblotting for total GR (Fig. 1B). No immunoreactivity toward GR is observed with preimmune sera (data not shown). As a control for specificity, we tested the ability of the antibody to detect phosphorylation-deficient GR S226A, stably integrated in U2OS cells (U2OS-hGR_{S226A}). The phospho-S226 antibody did not recognize GR S226A in either the absence or presence of Dex (Fig. 1B). Thus, the GR phospho-S226 antibody recognized its phosphorylation site specifically.

We also examined phosphorylation of endogenous GR in A549 cells. In contrast to what was observed in U2OS cells ectopically expressing hGR, GR phosphorylation at S226 in A549 cells increases only slightly upon treatment with Dex (Fig. 1B).

To further explore GR phosphorylation in both cell types, we examined the kinetics of GR phosphorylation at S226, S203, and S211 using antibodies specific for these phosphoserine residues. An antibody that recognizes GR independent of its phosphorylation state was used to determine the total amount of GR, and actin was used as a control for loading. As shown in Fig. 1C, phosphorylation at S226 in U2OS cells increased rapidly within the first hour of Dex treatment and remained high for 4 h, after which the signal decreased progressively from 6–12 h. Phosphorylation of S211 followed a pattern similar to that of S226 throughout the hormone treatment, whereas S203 had a higher basal level of phosphorylation and a less dramatic hormone induction (Fig. 1C). Thus, in U2OS ectopically expressing hGR, basal phosphorylation of S226 is low, and Dex-dependent phosphorylation at S226 is high, which mirrors that of S211.

In contrast, GR phosphorylation at S226 in A549 cells increases only slightly upon treatment with Dex (Fig. 1C, *right panel*) whereas the dynamics of hormone-dependent phosphorylation at S211 and the high basal level of phosphorylation at S203 are similar to that in U2OS-hGR cells. Thus, phosphorylation at S226 appears to vary between the two cell types, which may reflect differences in the availability or activity of GR kinases and phosphatases.

Ligands Effect GR Phosphorylation Differentially

We next tested a battery of ligands [Dex, cortisol, deooxycorticosterone, progesterone, progestin (R5020), 17-α-hydroxyprogesterone, aldosterone, R1881, testosterone, and dihydrotestosterone] with different potencies for their ability to induce GR phosphorylation. As shown in Fig. 2A, Dex, cortisol, deooxycorticosterone, progesterone, and progestin but not aldosterone, R1881, testosterone, or dihydrotestosterone promote S226 phosphorylation. In addition, RU486 induced S226 phosphorylation to levels similar to Dex in U2OS cells (data not shown). For the S211 site, phosphorylation was induced only by Dex, cortisol, and to a lesser extent, deooxycorticosterone. We have previously shown that phosphorylation at S211 was not induced by RU486 (16). Surprisingly, S203 phosphorylation was augmented by virtually all of the ligands examined, suggesting that S203 phosphorylation may reflect a nonselective effect of steroid localization. These results suggest that GR phosphorylation is differentially affected by agonist potency.

To test the influence of S211 and S226 phosphorylation on GR transcriptional activation, we assessed the activity of mouse mammary tumor virus (MMTV)-luciferase reporter in U2OS cell lines expressing wild-type (WT) or S226A hGR in the presence of 100 nM Dex, an inducer of higher phosphorylation at S211 relative to S226, or progesterone, a weaker inducer of S211 phosphorylation compared with S226 (Fig. 2A). As expected, Dex induced reporter gene activation in the WT GR cells, whereas progesterone did not. Interestingly, Dex initiated a more robust induction in the S226A line (Fig. 2B), in which even the suboptimal agonist progesterone modestly stimulated reporter gene activity. Thus, the relative level of phosphorylation at S211 *vs.* S226 is an important determinant of receptor activity and is consistent with the idea that S226 phosphorylation is inhibitory to GR transcriptional activation.

GR Hormone-Dependent Phosphorylation at S211 Is Affected by BAPTA-AM and Curcumin

Cellular signaling pathways potentially involved in GR phosphorylation were investigated by using pharmacological inhibitors of kinases and other signaling pathways (see Table 1). U2OS-hGR cells were treated for 1 h with the compounds, and GR phosphorylation at S203, S211, and S226 was determined after a 1-h Dex treatment. BAPTA-AM, an intracellular calcium-chelating agent, dramatically decreased hormone-de-

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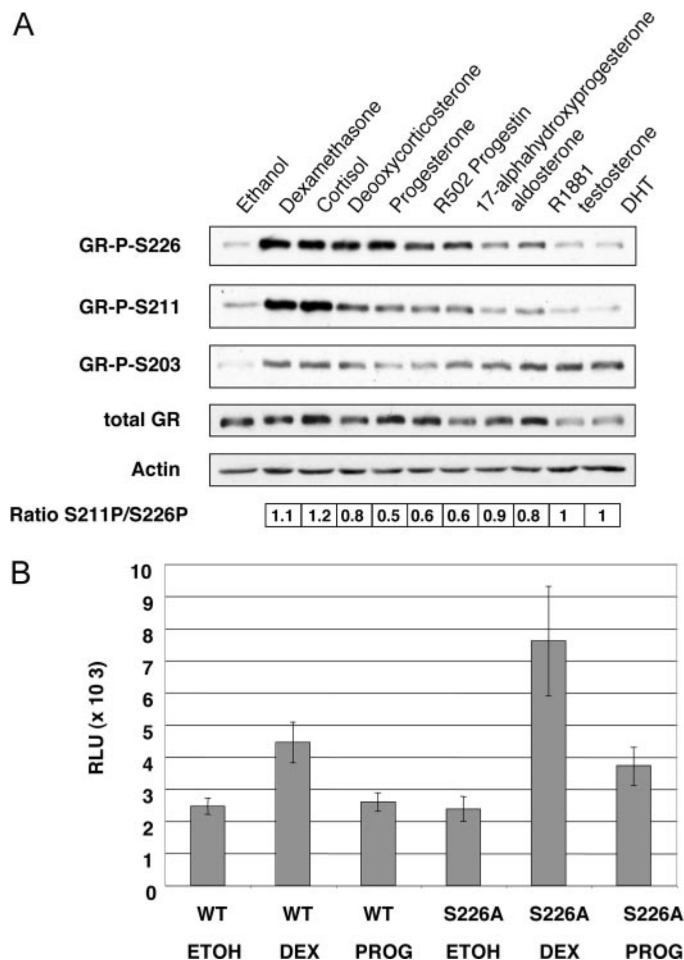

Fig. 2. Effects of Ligands on GR Phosphorylation and Transcriptional Activation

A, U2OS-hGR were treated with ethanol (−) or the ligands indicated (100 nM) for 1 h, and whole-cell extracts were prepared. Equal amounts of protein from each treatment were analyzed by immunoblotting with phospho-S226, phospho-S211, phospho-S203, total GR, or actin antibodies, reflective of the amount of total protein in each lane. The ratio of GR phosphorylation at S211 to S226 normalized to total GR is shown (S211P/S226P). B, U2OS-hGR WT or U2OS-hGR S226A (S226A) cells were transiently transfected with the MMTV-luciferase reporter construct along with pCMV-lacZ as an internal control. After 16 h, the cells were treated with ligands indicated (100 nM) for 1 h, conditions identical to those used for the immunoblot analysis, and luciferase activity was determined. DHT, Dihydrotestosterone; PROG, progesterone; RLU, relative light units.

pendent phosphorylation at the S211 (Fig. 3A). Curcumin, the yellow coloring agent in curry powder known as turmeric, which suppresses the nuclear factor-κB (NFκB) pathway through an unknown mechanism, inhibited GR phosphorylation at S211 at the highest concentration tested (50 μM) and also reduced phosphorylation at S203 and S226 to the level observed in the absence of hormone. DRB, a potent inhibitor of cyclinH/CDK8 and cyclin T/CDK9, did not decrease GR phosphorylation (Fig. 3A). Surprisingly, none of the other CDK or MAPK inhibitors (Table 1), significantly reduced phosphorylation at any of the sites under the conditions of the assay (data not shown), despite previous findings that cyclinA/CDK2 and MAPK phosphorylate GR activation function 1 (AF-1) *in vitro*, and mutations in the kinases or their inhibitors affected GR transcriptional activation (15, 17). This may reflect redundancy of the CDKs and MAPKs with respect to GR phosphorylation or alterations in the drugs or targets that render them insensitive to inhibition in U2OS cells. BAPTA-AM and curcumin also reduced GR S211 hormone-dependent phosphorylation in human A549 lung epithelial cells and in a rat hepatoma cell line (data not shown). Chelating extracellular calcium with EGTA had no effect on GR phosphorylation at any of the sites. Similarly, thapsigargin, which depletes intracellular calcium stores, had virtually no effect on GR phosphorylation, suggesting that the effect of BAPTA-AM on GR phosphorylation is independent of intracellular calcium levels.

We also examined whether BAPTA-AM, curcumin, and DRB alter Dex-dependent nuclear localization of GR by immunofluorescence (Fig. 3B). BAPTA-AM and curcumin, but not DRB, resulted in retention of GR in the cytoplasm and also reduced the activity of an MMTV-luciferase reporter (data not shown). This is

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Table 1. Inhibitors Examined for Effects on GR Phosphorylation

Inhibitor (Concentration)	Cellular Target
BAPTA-AM (40 μg/ml)	Intracellular divalent cation chelator
Cyclosporine A (CSA) (1 μg/ml)	Calcineurin inhibitor
Curcumin (50 μM)	Multiple inhibitory functions
DRB (100 μM)	CK2/CDK8/9 inhibitor
EGTA-AM (3 mM)	Extracellular Ca^{2+} chelator
Genistein (300 μM)	Tyrosine kinase inhibitor
Glybendamide (15 μM)	Cl^{-} channel blocker
HA1004 (45 μM)	cAMP/cGMP kinase inhibitor
IC261 (10 μM)	CK1 inhibitor
LY294002 (15 μM)	PI3 kinase inhibitor
Rapamycin (10 μM)	p70 S6 kinase inhibitor
Roscovine (7 μM)	p34 cdc2/cyclinB kinase inhibitor
SB202190 (35 μM)	p38 MAPK inhibitor
Staurosporine (0.5 μM)	General kinase inhibitor
Thapsigargin (2 μM)	ER Ca^{2+} pump inhibitor
Tunicamycin (2.5 μg/ml)	N-linked glycosylation inhibitor
U0126 (0.7 μM)	MEK1 and MEK2 inhibitor
Valinomycin (10 μM)	Ca^{2+} ATPase pump blocker
Wortmanin (0.1 μM)	PI3 kinase inhibitor

Bold lettering indicates compounds that affected GR phosphorylation; the remaining compounds had little or no measurable effect on GR phosphorylation at the sites examined. MEK, MAPK kinase; PI3, phosphatidylinositol.

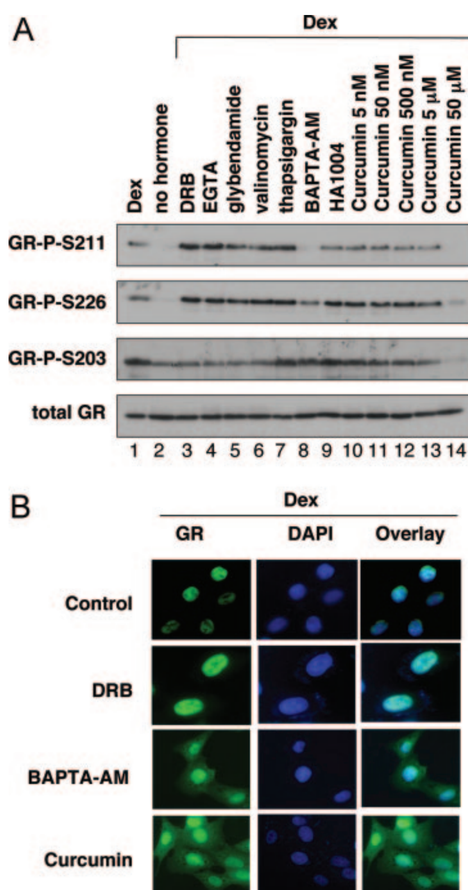

Fig. 3. Signaling Pathways Involved in GR Phosphorylation

U2OS-hGR cells were pretreated for 1 h with indicated compounds at the concentrations found in Table 1, and GR phosphorylation at S203, S211, and S226 was determined after a 1-h Dex treatment. Equal amounts of protein from each treatment were analyzed by immunoblotting with phospho-S226, phospho-S211, phospho-S203, or total GR antibodies. B, Subcellular distribution of GR upon treatment with BAPTA-AM, curcumin, and DRB. U2OS-hGR cells, treated exactly as above, were fixed, and the subcellular location of GR was examined by indirect immunofluorescence using a total GR antibody. The data shown are from a single experiment that is representative of at least three independent experiments. DAPI, 4′,6-Diamidino-2-phenylindole.

consistent with a previous report demonstrating that BAPTA-AM inhibited hormone-dependent GR nuclear import (23). This suggests that reducing GR hormone-dependent phosphorylation decreases localization of GR to the nucleus even in the presence of Dex. Alternatively, reduced GR phosphorylation may be secondary to the failure to localize to the nucleus. Additional experiments will be required to distinguish between these possibilities.

Molecular Modeling of GR Polypeptides Encompassing the S211 Phosphorylation Site

To gain insight into the potential local structural changes in GR upon phosphorylation at S211, we performed extensive global energy minimization simulations with two peptides, bearing a phosphorylated *vs.* nonphosphorylated S211 (Q_{195}DLEFSSGSPGKETNE$\mathbf{S_{211}}$PWRSDLLI$_{220}$) (Fig. 4). The predicted conformation of the unmodified peptide exhibits a largely random conformation (Fig. 4A), consistent with biophysical measurements of the GR AF-1 structure (24). Interestingly, phosphorylation of S211 induces a marked trend toward a more structured conformation with the peptide adopting a helical structure on both sides of the phosphorylation site (Fig. 4B). This suggests that phosphorylation of S211 can confer structure to the GR AF-1 domain. It is also worth noting that many of the conformations in the phosphopeptide exhibited a hydrogen bond interaction between the phosphate moiety on the S211 and an adjacent arginine at residue 214 (R214), which in turn makes contact with glutamic acid 207 (E207) (Fig. 4C). Interestingly, E207 and R214 are conserved among receptor from diverse species, and R214 is also part of the consensus sequence for CDK phosphorylation [S-(P)-P-X-R/K]. This implies that flanking residues in the kinase consensus site might have evolved to contribute to the stabilization of local structure upon phosphorylation.

In close proximity to the S211 phosphorylation site are three amino acid residues within the GR AF-1 (E198, F220, and W213) the simultaneous disruption

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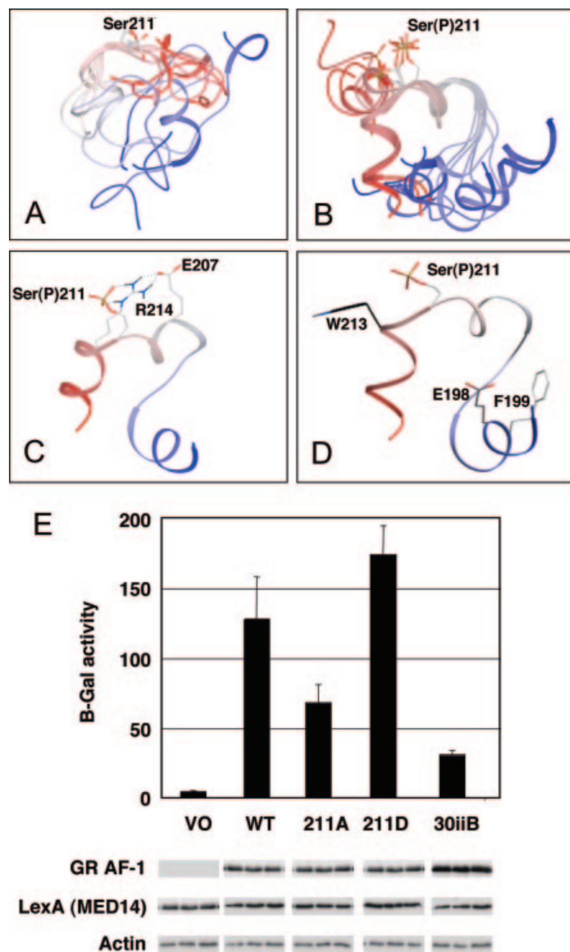

Fig. 4. GR Phosphorylation at S211 Results in Structural Changes and Modulates Interaction with MED14 (Vitamin D Receptor Interacting Protein 150)

Representative low-energy conformations of the (A) non-phosphorylated (WT) and (B) phosphorylated (P-S211) peptide spanning the S211 site. Peptide is colored *blue* (N-term) to *red* (C-term) and S211 is depicted. Note that the phosphorylated peptide displays a more structured conformation around the phosphorylated residue, with the peptide adopting a helical structure on both sides of the phosphorylation site. C, Interaction of P-S211with R214. Residues P-S211, R214, and E207 are shown forming a hydrogen bond network that is displayed as *dots* between donor and acceptor atoms. D, Location of E198, F220, and W213, which flank the S211 phosphorylation site and have been shown to be essential for transcriptional activation at certain GREs. E, Phosphorylation-dependent interaction of MED14 with GR AF-1 derivatives in yeast two-hybrid assay. $MED14_{1214–1434}$ expressed in yeast as a fusion protein to the LexA DNA-binding domain (pEG202) was analyzed for its ability to interact with the WT GR AF-1 (WT), GR 30IIB (30IIB), which harbors three point mutations in AF-1, and S211A and S211D fused to the HA epitope and B42 activation domain in a galactose-inducible expression vector (pJG4/5). Strength of interaction is determined by quantitative liquid β-galactosidase assays after a 12-h incubation at 30 C in galactose containing media. Data represent the average β-galactosidase activity of three independent clones, and *error bars* are the SD. Western blots of extracts from strains expressing the indicated HA-GR fusions were performed using a HA-specific monoclonal antibody, a polyclonal antibody to LexA to detect the LexA-Med14 derivatives, and actin as loading control. Shown is protein expression from the three independent clones used in the assay. B-Gal, β-Galactosidase; VO, vector only.

of which (30IIB mutant) reduces receptor transcriptional activation (25). We therefore examined the location of these amino acids on the predicted structure. Interestingly, these residues localize within the helical portions of the GR polypeptide formed upon phosphorylation (Fig. 4D), indicating that the regions surrounding S211 are of critical importance to GR transcriptional activation perhaps because they contribute to the formation of the putative protein-protein interaction surfaces.

GR S211 Phosphorylation Modulates Interaction with MED14 (Vitamin D Receptor Interacting Protein 150)

Previous studies from our laboratory demonstrated that MED14 associates with the GR AF-1 (26). To determine whether GR phosphorylation at S211 affects its interaction with MED14, we examined the effect of S211 substitution with an alanine (S211A) and an aspartic acid (S211D), which potentially mimics the phosphorylated state of GR, on MED14 binding in a yeast two-hybrid assay. GR ectopically expressed in yeast is phosphorylated at the same sites observed in mammalian cells (15). The GR phosphorylation site derivatives were expressed in yeast, along with MED14 (Fig. 4E, *lower panels*) and a β-galactosidase reporter, and their interaction was determined.

As expected, the GR N-terminal activation domain interacted with MED14, whereas the mutation (30IIB) significantly reduced the interaction (26). A similar, albeit smaller, decrease was observed with the GR S211A mutant (Fig. 4E). In contrast, the phosphomimetic S211D substitution increased GR interaction with MED14. This suggests that GR phosphorylation at S211 modulates its interaction with MED14.

GR Phosphorylation Site Mutations Differentially Affect GR Target Gene Expression

To better understand the effect of phosphorylation on GR-dependent gene expression, we measured the mRNA accumulation of established GR target genes glucocorticoid-induced leucine zipper (GILZ), ladinin 1 (LAD1), IGF-binding protein 1 (IGFBP1), and interferon regulatory factor 8 (IRF8) in U2OS cells that stably express GR derivatives bearing serine to alanine substitutions at S226 (U2OS-hGR_{S226A}) and S211 (U2OS-hGR_{S211A}) at levels similar to WT GR in U2OS-hGR cells (11, 21, 27). U2OS-hGR_{S226A} is a pool of clones stably expressing S226A, whereas the U2OS-hGR_{S211A} line is a single clone. We have verified our findings with a pool stably expressing S211A (data not shown). After a 2-h Dex treatment at the indicated

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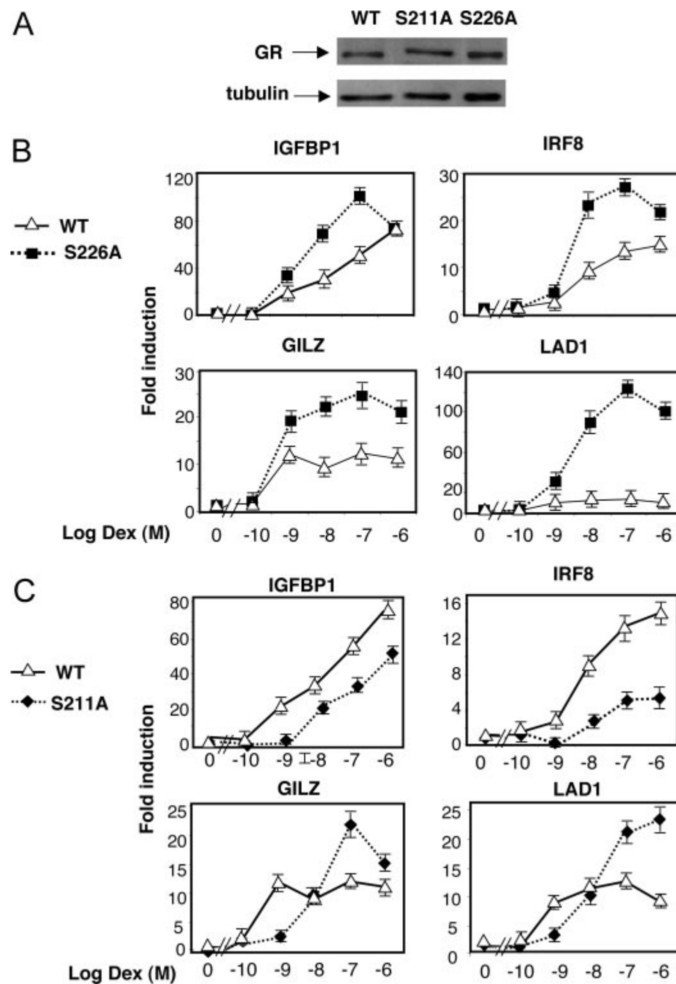

Fig. 5. GR Target Genes Are Differentially Responsive to Mutations in the GR Phosphorylation Sites, S226 and S211

A, GR constructs S211A and S226A stably expressed in U2OS cells were analyzed by immunoblotting with antibodies to GR and actin as an internal control for loading. B and C, U2OS cells stably expressing GR WT, GR S226A, or S211A were treated with ethanol vehicle (0) or the indicated amount of Dex for 2 h, and total RNA was analyzed by real-time PCR as described in *Materials and Methods*. The values indicate expression of target genes normalized to Rpl19 RNA and presented as fold induction relative to vehicle-treated cells. The data shown are from three independent experiments. The *error bar* indicates the SD.

doses, total RNA was isolated and subjected to qPCR using gene-specific primer pairs (11). We first examined the expression of GR target genes in U2OS cells harboring the S226A mutant relative to cells with WT GR, and found it to be enhanced for all genes tested (Fig. 5A). For example, the induction of LAD1 by GR S226A at 10 nM Dex is 6-fold greater than that by the WT, and the induction of IGFBP1, IRF8, and GILZ is 2-fold over WT. This establishes that S226 phosphorylation inhibits glucocorticoid induction of the four endogenous GR targets examined.

Quantitative analysis revealed two patterns of dose-dependent accumulation of mRNA in U2OS-hGR_{S211A} as compared with cells expressing the WT GR (Fig. 4B). Dex-dependent induction of IGFBP1 and IRF8 mRNAs by S211A was reduced relative to WT at all hormone concentrations tested, which shifts the dose-response curve to the right. This change may suggest that the S211A mutant is less efficient at engaging in the associations with cofactors, such as MED14, necessary for transcriptional activation of IGFBP1 and IRF8. This is consistent with our previous findings that IRF8 and IGFBP1 required MED14 for activation by GR (11). In contrast, induction of GILZ and LAD1 by S211A is lower than that by WT at Dex concentrations below 10 nM, and similar to or even higher at hormone concentrations above 10 nM. This suggests that distinct mechanisms are employed by GR in the induction of GILZ and LAD1 at low *vs.* high Dex concentrations, which is affected by receptor phosphorylation. Interestingly, our previous analysis indicates that GILZ, for example, is largely MED14 independent, at least at 100 nM Dex (11). This effect was confirmed in three independent clones as well as a pooled line (data not shown). These findings indicate that the

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effect of GR S211 phosphorylation is gene specific and, in some cases, may also be dependent upon the amount of activated receptor.

We also examined whether GR phosphorylation site mutations affected the ability of GR to repress gene expression using the U2OS cell system. Seven GR target genes were examined that were repressed by GR in U20S cells (I. Rogatsky, unpublished data). These include cMyc, ephrin, Fra, cJun, JunB, Smad7, and PAC1. As with activation, the lack of GR phosphorylation at S211 results in gene-specific effects on repression by GR. For example, the ability of the S211A mutant to repress JunB was severely compromised compared with wild-type GR, whereas cMyc and Smad7 was only moderately affected, and repression of ephrin, Fra, and cJun was largely insensitive to the S211A mutation (Fig. 6, B and C). Unexpectedly, a majority of these genes were only minimally affected by the S226A mutation (Fig. 6, A and B). Interestingly, Pac 1, which is modestly repressed by the wild-type GR, in fact was induced by the S211A and to a lesser extent by the S226A mutants compared with WT GR (Fig. 6C). Basal levels of the repressed target genes were virtually unchanged between phosphorylation site mutants and wild-type lines. Thus, phosphorylation appears to affect the magnitude of repression by GR in a gene-selective manner, and in the case of Pac1, can convert a repressed gene to one that is now activated by GR. This may suggest that common surfaces within GR affected by phosphorylation may be responsible for both positive and negative regulation.

DISCUSSION

It has been proposed that hormone-dependent phosphorylation of steroid receptors is a mechanism to modulate receptor transcriptional responses (14, 28–30). Here we provide evidence that GR phosphorylation at S211 and S226 can indeed affect receptor transcriptional activation in a gene-specific manner. Furthermore, this regulation can be both positive and negative, and the relative level of phosphorylation at S211 *vs.* S226 is an important determinant of receptor

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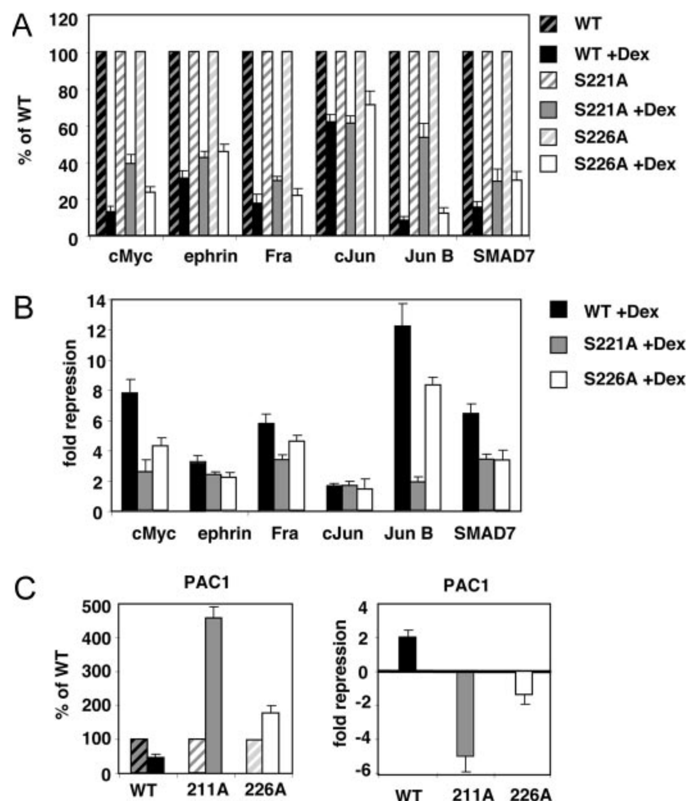

Fig. 6. Repression by GR Is Phosphorylation Sensitive

U2OS cells stably expressing GR WT, GR S211A, or S266A were treated with ethanol vehicle or 100 nM Dex for 2 h, and total RNA was analyzed by real-time PCR. The values indicate expression of target genes normalized to RPL19 mRNA levels and presented relative to the expression of vehicle-treated cells, which was set as 100%. A, The level of hormone-dependent repression over the untreated control in each clone is shown. B, The percent repression of each gene by GR S211A and S226A relative to that of WT. C, The level of hormone-dependent repression (*left*) and the percent repression of PAC1 by the GR mutants relative to WT is shown. The *error bar* represents the SD from a single experiment performed in triplicate. This experiment was repeated twice, and the same patterns were observed. SMAD.

activity. Thus, comparatively higher phosphorylation at S211 relative to S226 correlates with GR nuclear localization and greater transcriptional activation and *vice versa*. This is consistent with previous findings that phosphorylation of GR S226 by JNK specifies receptor nuclear export under conditions of hormone withdrawal, a condition that favors low S211 and high S226 phosphorylation levels (19, 20). Given that the glucocorticoid induction of all GR target genes examined (GILZ, IGFBP1, IRF8, and LAD1) was increased in the GR S226A-expressing cells (Fig. 5A), we conclude that phosphorylation at S226 reduces GR transcriptional activation through some general mechanism such as the enhanced nuclear export. A careful kinetic examination of nuclear import and export of the GR phosphorylation site mutations will help to address mechanism. It should be noted that our previous work has shown that S226A phosphorylation enhances hormone-dependent phosphorylation at S211 (21), which could also contribute to increased transcriptional activation of GR S226A.

In contrast, the impact of S211 phosphorylation on GR target gene expression is gene specific (Fig. 5B) and paralleled the requirements for MED14 in gene activation by GR (11). Structure simulations reveal that phosphorylation of GR at S211 may induce the receptor to acquire a helical conformation around the site (Fig. 4, A and B). In addition, S211 phosphorylation enhanced receptor interaction with MED14 (Fig. 4E), further reinforcing the idea that phospho-S211 may comprise a part of a cofactor-binding surface. Indeed, disruption of this interaction by the S211A mutation results in a shift of the ligand dose-response curve in the case of IGFBP1 and IRF8 (Fig. 4), genes the glucocorticoid induction of which is MED14 dependent. Modulation of the dose-response curve by changing GR-MED14 interaction would be predicted by important work from the Simons laboratory (31). In addition, elegant studies by Kumar *et al.* (24) have shown that acquisition of structure in the GR AF-1 can lead to enhanced interaction with the TATA-binding protein (TBP). It will be interesting to test whether this interaction is also modulated by receptor phosphorylation.

Phosphorylation-dependent coregulator recruitment is not limited to GR. Classic studies with CREB have shown that phosphorylation by protein kinase A results in binding of CBP (32). Hammer *et al.* (33) revealed that phosphorylation of SF-1 by MAPK enhanced coregulator recruitment. Likewise, estrogen receptor (ER)α phosphorylation of S118 facilitates interactions with (SF) 3ap120, a splicing factor (34), with steroid receptor coactivator 3 (35), as well as with stromelysin-1 platelet-derived growth factor-responsive element-binding protein that represses ER activity (36, 37).

Unlike IRF8 and IGFBP1, the glucocorticoid induction of which was lessened by the S211A mutation across the hormone dose curve, activation of GILZ and LAD1 was impaired at a low concentration of Dex ($<$10 nM), but equal to or greater than that of WT at greater than 10 nM Dex (Fig. 5). GILZ harbors multiple glucocorticoid response elements (GREs) that contribute to activation by GR (38), whereas IGFBP1 (39) and IRF8 (11) have noncanonical GREs that display low GR occupancy relative to GILZ. We previously suggested that weak GR binding to DNA might bias genes toward MED14-dependent RNA polymerase II recruitment, whereas high GR recruitment bypasses this requirement (11). Consequently, under low hormone concentrations GR occupancy at GILZ is low, and its expression is more dependent on S211 phosphorylation and MED14 recruitment (Fig. 5B), whereas at higher hormone concentration GILZ induction becomes independent of phospho-S211 and MED14. In contrast, genes like IRF8, which are regulated through weak GREs, would operate entirely through the S211 phosphorylation- and MED14-dependent pathway (Fig. 7). We conclude that the role of GR phosphorylation at S211 is largely to facilitate the GR-MED14 interaction and transcriptional enhancement.

From our analysis, we can begin to classify GR target genes into phosphorylation-dependent and -independent behaviors (Fig. 6B). The S211 phosphorylation-dependent genes are expected to be MED14 dependent and would include those at which GR occupancy is modest by virtue of a low GRE number or affinity (*e.g*. IGFBP1), or under suboptimal conditions, such as low hormone levels (*e.g*. GILZ). In contrast, S211 phosphorylation-independent genes are largely MED14 independent and would include those with multiple high-affinity GREs, as exemplified by GILZ under conditions of high hormone and receptor levels. Notably, phosphorylation-independent genes may behave like phosphorylation-dependent genes under conditions of fractional GR occupancy as a result of low hormone and/or receptor levels.

Because LAD1 induction by GR S211A mirrors that of GILZ, one would expect that LAD1 would harbor multiple high-affinity GREs. However, we have been unable to locate the GR binding sites in LAD1 by chromatin immunoprecipitation scanning of approximately 5 kb of DNA upstream of the start site of transcription. In light of the recent analysis of ER binding throughout the genome, this may not be surprising given that a majority of regulatory regions bound by the receptor are neither upstream nor nearby the start site of transcription (40). Additional studies will have to be performed to determine the location and nature of the LAD1 GREs.

These studies reveal one potential mechanism of how GR phosphorylation may modulate gene expression. They suggest that some GR-responsive genes will be hard wired and inherently GR phosphorylation dependent because of the restricted number and affinity of their GREs (*e.g*. IGFBP1). GR occupancy could also be influenced by adjacent transcription factor-binding sites, such as, for example, binding of FoxA1 for ER recruitment (41). In contrast, other genes (*e.g*. GILZ) may differentially respond to GR phosphorylation, due to multiple high-affinity GREs capable of

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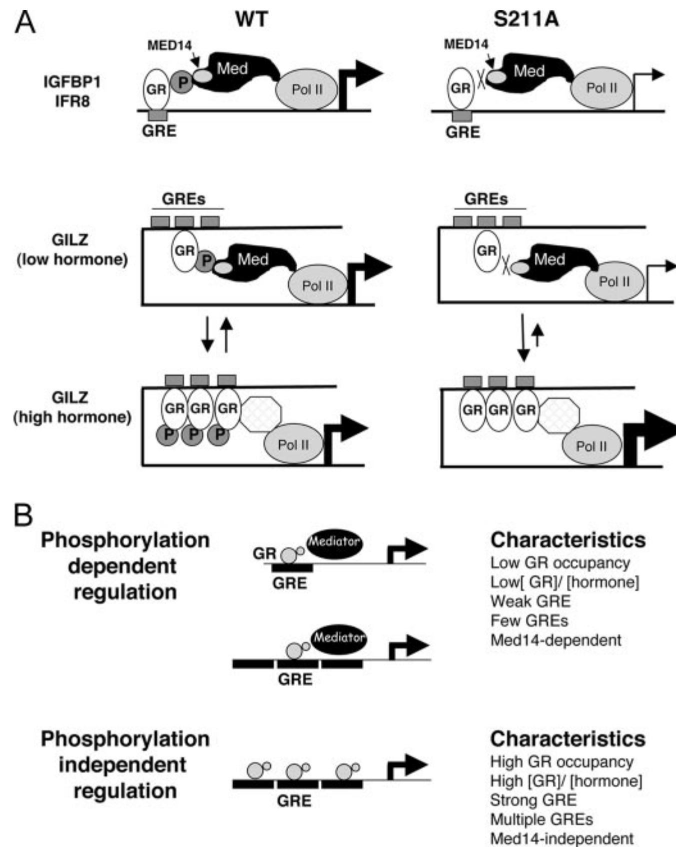

Fig. 7. Interplay among GR Phosphorylation, GRE Occupancy, and Cofactor Recruitment Regulates GR Target Gene Response
A, Models for GR S211 phosphorylation-dependent and -independent gene expression. For IGFBP1 and IRF8, GR S211 phosphorylation (*encircled* P) facilitates recruitment of the Mediator complex via MED14, which, in turn, recruits the RNA polymerase II (Pol II) and the basal transcriptional machinery. The S211A mutation would reduce this interaction and transcriptional activation. A similar scenario is envisioned at GILZ under low hormone concentrations when only a fraction of the GREs are occupied by GR. At high hormone concentrations, GR will occupy all of the GILZ GREs and bypass the need for the MED14 and S211 phosphorylation by recruiting Pol II through another cofactor (*hatched*). B, GR S211 phosphorylation- and MED14-dependent genes are those with an inherently modest GR occupancy due to low affinity or number of GREs. Genes independent of S211 phosphorylation and MED14 contain multiple and/or high-affinity GREs capable of high GR occupancy under conditions of high hormone and/or receptor levels. Note that genes independent of S211 may display the behavioral characteristics of S211 phosphorylation-dependent genes under conditions of fractional GR occupancy as a result of low hormone and/or receptor levels.

wide-ranging GR occupancy states. This model may explain the often-conflicting data on the role of phosphorylation in GR function, which relied heavily on cell-based transient assays using synthetic reporter genes containing multiple high-affinity GREs and saturating receptor and hormone levels, all of which would favor a phosphorylation-independent response (42–44). This model is also consistent with early findings from Bai and Weigel (45) that a mutation of a phosphorylation site in the chicken progesterone receptor reduced transcriptional activity and that the magnitude of this effect was dependent upon the level of receptor expression. Elegant work from the laboratory of Alarid and associates (46) has shown that high nuclear concentrations of ERα in breast cancer cells can bypass the ligand requirement for activated transcription, and that this is independent of ER phosphorylation. Hence, increased receptor occupancy at a target gene, which in this case is driven by increased receptor expression, overrides the need for receptor phosphorylation. Likewise, important studies from the laboratory of Stallcup and co-workers (47) have shown that synergy among coactivators (*e.g*. GRIP1, CARM1, and PRMT1) occurs only when receptor levels are low, suggesting that different mechanisms of gene activation occur at high and low receptor concentrations.

Such an occupancy model for phosphorylation-dependent gene expression may hold true for other nuclear receptors and transcription factors, especially those that display cell type-specific patterns of phosphorylation (48). For example, signal transducer and activator of transcription 1 phosphorylation by IκB kinase ϵ in response to interferon β treatment has recently been shown to influence gene expression and signal transducer and activator of transcription 1 occupancy of select interferon-stimulated genes (49).

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Multiple androgen-responsive elements control prostate-specific antigen (PSA) activation by the androgen receptor (AR) (50). Depending on the level of AR expression or ligand concentration, PSA induction could be either dependent or independent of AR phosphorylation. This could explain the complex pattern of PSA expression (51, 52) as a function of AR and ligand concentration observed, for example, in androgen-independent prostate cancer where AR levels are often elevated (53, 54). Recently, we have found that liver X receptor (LXR)α phosphorylation has a greater impact on LXR target genes with noncanonical LXR binding sites, rather than those with strong consensus LXR-response elements (55). Thus, it appears that receptor levels and hormone strength, along with intracellular cofactor concentration, can set the stage for gene regulation through receptor phosphorylation. We also provide the evidence that GR phosphorylation plays a gene-selective role in transcriptional repression (Fig. 6). Whether receptor levels and cofactors such as MED14 play roles in repression remains to be elucidated. The interplay among GR phosphorylation, cofactor recruitment, and GRE type and/or abundance has likely evolved to adjust GR-dependent gene regulation to changing cellular needs.

MATERIALS AND METHODS

Antibody Production

The phosphopeptide, which corresponds to the following sequence in hGR: $_{218}$LLIDENLLS^{PO4}PLAG$_{230,}$ was synthesized by Anaspec, Inc. (San Jose, CA). A cysteine residue was added to the N terminus of the peptide to facilitate chemical cross-linking. The peptide was coupled to KLH and used to immunize rabbits (Covance Research Products, Inc., Denver, PA). Sera from immunized rabbits were tested for antibody titer and specificity for the phosphorylated peptide was tested by ELISA. High-titer antibodies were further tested on hGR expressed in U2OS by immunoblotting.

Cell Culture and Preparation of Cell Extracts

The human lung carcinoma cell line A549 (CCL-185) containing endogenous GR and the human osteosarcoma cell line U2OS (HTB 96) lacking endogenous GR were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM (Cellgro; Mediatech, Inc., Manassas, VA), supplemented with either 5% or 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), respectively, 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin (Cellgro). Generation of stable U2OS cell lines ectopically expressing hGR (wild-type and phosphorylation site mutants, S203A, S211A, and S226A) was performed as previously described. Individual neomycin-resistant clones and pools of clones were isolated and assayed for hGR expression by indirect immunofluorescence and immunoblotting with HA- and GR-specific antibodies. Clones homogeneously expressing HA-hGR were maintained at 500 μg/ml Geneticin.

Extracts for immunoblotting were prepared from a subconfluent 10-cm plate of A549 and U2OS-hGR cells treated with 100 nM Dex or equal volume of the ethanol vehicle 1 h before lysis. Cells were placed on ice, washed twice in PBS, lysed in 0.5 ml of buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol, and additional protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 8 mM sodium pyrophosphate, 1 μg/ml leupeptin, pepstatin A, and aprotinin (Roche, Indianapolis, IN). Lysates were centrifuged at 12,000 rpm for 15 min at 4 C. The soluble supernatants were normalized for total protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), and the samples were boiled for 3 min in 2× sodium dodecyl sulfate sample buffer and stored at −20 C.

Immunoblotting

Cell extracts or immunoprecipitates containing GR were separated by 10% SDS-PAGE and were transferred to Immobilon paper (Millipore Corp., Bedford, MA) at 110 V for 80 min in Tris-glycine transfer buffer. The membranes were blocked overnight in 5% BSA in Tris-buffered saline (TBS, pH 7.4; blocking solution) at 4 C, and then were incubated in the blocking buffer with primary antibody at room temperature (RT) for 2–4 h using 1:1,000 dilution of serum for phospho-S226 (Ab459) and phospho-S211 (Ab353), and 1:10,000 dilution of serum for phospho-S203 (Ab221). Endogenous GR was detected using the N499 polyclonal antibody, raised against residues 1–499 of the hGR. The membranes were washed three times for 10 min in TBS/0.1% Triton X-100 and twice in TBS and incubated for 1 h at RT with 0.2 μg/ml protein A conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD). For blots using the anti-HA-tag monoclonal antibody (α-HA; Roche), an horseradish peroxidase-conjugated goat-antimouse IgG secondary antibody was used. Blots were then washed three times for 10 min in TBS-0.1% Triton X-100 and twice in TBS and developed using enhanced chemiluminescence according to manufacturer's instructions (GE Health Sciences, Piscataway, NJ). Quantitative analysis of immunoblots was performed using the National Institutes of Health Image software package (version 1.62).

Immunofluorescence

U2OS-hGR cells were cultured in phenol red-free DMEM containing 10% charcoal-stripped fetal bovine serum on coverslips coated with poly-D-lysine and were treated with Dex for 1 h. Cells were fixed in cold acetone (−20 C) for 15 min, air-dried, and incubated in PBS containing 2.5% BSA for 1 h to block nonspecific protein binding. Cells were incubated with primary antibodies in blocking solution for 1 h at RT, washed five times in PBS-0.1% Triton X-100, followed by incubation with goat antimouse or goat antirabbit fluorescein-conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) diluted in blocking solution, for 1 h at RT. Secondary antibody was removed by washing the cells five times in PBS-0.1% Triton X-100 and three times in PBS. Coverslips were mounted onto Citifluor (Ted Pella, Redding, CA), and the fluorescein signal was visualized and photographed using a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc., Thornwood, NY).

Peptide Simulations

Modeling of peptides was performed using ICM software (MolSoft, La Jolla, CA) as previously described (56). Global energy minimization simulations were performed with two peptides, one with S211 phosphorylated and one without: Q$_{195}$DLEFSSGSPGKETNE$\mathbf{S_{211}}$PWRSDLLI$_{220}$. For each peptide, 40 parallel independent Biased Probability Monte Carlo global energy optimizations were performed starting from randomized conformations. The simulations of each pep-

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tide were terminated after 200 million energy evaluations. During stimulations, low-energy conformations were collected, merged, and sorted by energy, and redundant conformations were eliminated.

Real-Time PCR

Total RNA from U20S-hGR cells was extracted, and cDNA was synthesized and amplified on a LightCycler (Roche) with primers for the GILZ, LAD1, IGFBP1, and IRF8 as described elsewhere (11). The following primer pairs were used to profile the GR-repressed genes: cMyc, forward (F), 5′-TGAACCAGAGTTTCATCTGCG-3′; reverse (R), 5′-TTCTCTGAGACGAGCTTGGC-3′; Fra1, F, 5′-CATCGCAAGAGTAGCAGCAG-3′; R, 5′-AGGAGACATTGGCTAGGGTG-3′; Ephrin1, F, 5′-CTTCACACCTTTCACCCTGG-3′; R, 5′-CAGTCACCTTCAACCTCAAGC-3′: cJun, F, 5′-TGCAAAGATGGAAACGACC-3′; R, 5′-TGCTCTGTTTCAGGATCTTGG-3′: JunB, F, 5′-AACATGGAAGACCAAGAGCG-3′; R, 5′-TCTTCACCTTGTCCTCCAGG-3′; SMAD7, F, 5′-TGCTGTGAATCTTACGGGAAG-3′; R, 5′-AATCCATCGGGTATCTGGAG-3′: PAC1, F, 5′-ATACCTCATGCAGATCGCC-3′; R, 5′-ACAGCACCTGGGTCTCAAAC-3′.

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