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Glucocorticoid Receptor Phosphorylation Modulates Transcription Efficacy through GRIP-1 Recruitment[†]

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ABSTRACT: The role of GR phosphorylation in modulating GR-mediated transcription is not fully understood. Here we show that the hGR is rapidly phosphorylated at S211 and S226 in response to the synthetic agonist dexamethasone (dex) in COS-1 cells. Using a triple phosphorylation mutant hGR construct, we demonstrate that phosphorylation at one or more S residues (from S203, S211, and S226) is required for maximal hGR-mediated transcriptional activation on the MMTV promoter in response to dex in COS-1 cells, but that this effect is promoter selective. Phosphorylation at these residues does not affect unliganded or agonist-induced hGR degradation, suggesting that the mechanism whereby hGR phosphorylation at these residues regulates GR-mediated transactivation via a GRE does not involve changes in GR half-life. We have previously shown a direct correlation between efficacy for transactivation and interaction of the hGR with glucocorticoid receptor interacting protein-1 (GRIP-1). Here we show by pull-down assays in the absence and presence of glucocorticoid response elements (GREs) that phosphorylation of the hGR is required for GR−GRIP-1 interaction. Chromatin immunoprecipitation (ChIP) assays revealed that hGR phosphorylation at one or more S residues (from S226, S211, and S203) is required for the recruitment of GRIP-1 to the synthetic MMTV promoter as well as to the endogenous GRE-containing glucocorticoid-induced leucine zipper (GILZ) promoter in intact COS-1 cells, but not for nuclear localization. Our results support the conclusion that phosphorylation at S203, S211, and/or S226 of the hGR is required for a maximal transcriptional response via the synthetic MMTV and endogenous GILZ GREs in COS-1 cells, to enable recruitment of GRIP-1 to the hGR.

Glucocorticoids (GCs)[1] play an important role in most physiological processes, including inflammation, homeostasis, stress, and development (*1*, *2*). They mediate their actions through binding to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily, which is a ligand-activated transcription factor (as reviewed in ref *3*). In the absence of ligand, the GR forms a complex with inhibitory proteins, including heat shock protein 90 (HSP90), which keeps the GR localized in the cytoplasm and maintains a conformation suitable for ligand binding. Once the ligand binds, the GR undergoes a conformational change, dissociates from the HSP90 complex, and translocates to the nucleus where it can target genes through direct DNA binding to glucocorticoid response elements (GREs), or via tethering to other transcription factors, thereby activating or repressing gene transcription (as reviewed in ref *4*).

The GR, as well as other members of the steroid receptor superfamily, undergoes post-translational modifications, including phosphorylation (*5*−*10*), acetylation (*11*−*13*), and ubiquitylation (*14*−*17*). In the absence of ligand, the GR is basally phosphorylated but becomes hyperphosphorylated in response to agonist binding (*18*). Three important serine residues have been identified in the hGR, namely, S203, S211, and S226, also conserved at similar positions in the mGR (*18*). All three of these serine residues reside within the N-terminal domain of the GR, which contains the constitutive transcriptional activation function 1 (AF1) domain, suggesting a role for phosphorylation in transcription (*19*, *20*). Reports in the literature about whether phosphorylation of the GR plays a role in regulating transcription are varied, suggesting that phosphorylation has an only modest effect (*21*), results in a decrease in transcription (*2*), or has variable effects depending on the specific gene investigated (*22*, *23*). Taken together, it appears that the effect on transcription is dependent on the particular residue(s) phosphorylated as well as being dependent on cell, promoter, and GC concentration (*2*, *21*−*23*).

In attempts to determine the mechanism whereby GR phosphorylation influences transcription, several steps in the GR signaling pathway have been implicated, including nuclear trafficking, protein stability, and protein−protein interactions (recently reviewed in ref *18*). Wang et al. demonstrated that hormone stimulation leads to the accumulation of hGR phosphorylated at S211 in the nucleus, whereas hGR phosphorylated at S203 remains cytoplasmic, suggesting a role for phosphorylation at S211 in nuclear import in U2OS cells (*24*). However, mutation of up to eight phosphorylation sites of the mGR did not affect ligand-mediated nuclear import of the GR in COS-1

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[1]Abbreviations: GCs, glucocorticoids; GR, glucocorticoid receptor; dex, dexamethasone; GRE, glucocorticoid response element; GRIP-1, glucocorticoid receptor interacting protein-1; MMTV, mouse mammalian tumor virus; ChIP, chromatin immunoprecipitation; SEM, standard error of the mean.

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cells (*22*). It has been shown that phosphorylation of the progesterone (*25*) and estrogen receptors (PR and ER, respectively) influences receptor localization in the absence of ligand and that phosphorylation of the liganded androgen receptor (AR) is required for nuclear export (recently reviewed in ref *18*). However, nuclear import does not appear to be affected by agonist-induced phosphorylation of the PR, AR, or ER. These results suggest that the role of agonist-induced phosphorylation in steroid receptor subcellular trafficking may be receptor- and cell-specific or depend on which particular serine residue(s) within a particular receptor is phosphorylated.

Several lines of evidence point to receptor phosphorylation playing a role in receptor protein degradation both in the absence and in the presence of ligand. In the literature, there are, however, conflicting reports about whether phosphorylation is required for GR nuclear import, but there is evidence that mGR phosphorylation plays a role in stabilizing the mGR protein. Webster et al. showed that the simultaneous mutation of eight phosphorylation sites on the mGR stabilized the unliganded GR protein, resulting in its slower degradation in COS-1 cells (*22*). Furthermore, the increased rate of mGR protein degradation typically found upon dex stimulation (*17*, *26*) was shown by Webster et al. to be completely abolished in the mGR containing mutations at eight serine residues (*22*). The concept of modulation of steroid receptor degradation via phosphorylation is not limited to the GR, as similar observations have been made for the AR and PR (*7*, *14*, *16*). Phosphorylation of the PR at S400 enhances degradation of the unliganded PR (*25*), whereas phosphorylation at S294 enhances degradation of the liganded PR (*7*), indicating a complex mechanism involving serine-specific phosphorylation on the stability of the unliganded or liganded PR. In contrast to the GR, agonist binding to the AR leads to the increased stability of the receptor, which is believed to be mediated via agonist-induced AR phosphorylation (*27*). Thus, steroid receptor phosphorylation is shown to play a role in the stability of the unliganded and liganded receptor, where the effect on the liganded receptor may be a mechanism for regulating transcription efficacy. However, as for the effect of receptor phosphorylation on subcellular trafficking, these effects may be receptor- and cell-specific or depend on which particular serine residue(s) within a particular receptor is phosphorylated.

Aside from the studies on nuclear translocation and degradation of the GR mentioned above, recent studies also demonstrate that differences in the phosphorylation status of the GR influence the interaction of the GR with other proteins, including tumor suppressor gene protein, components of the mediator complex, and CBP/p300 (*2*, *23*, *28*, *29*). In U2OS cells, the tumor suppressor gene protein (TSG101) binds preferentially to the unphosphorylated form of the unliganded expressed hGR and thereby stabilizes the unliganded GR against degradation (*28*). Using a yeast two-hybrid screening, it was found that vitamin D receptor-interacting protein 150 (DRIP150 or MED14), which forms part of the mediator complex, binds to the hGR AF-1 domain and that this interaction enhances GR transactivation (*30*). It was later shown that mutation of S211 resulted in a 50% decrease in the level of binding of MED14 to the expressed hGR (*23*), indicating a possible role for GR phosphorylation in the recruitment of the mediator complex in U2OS cells. Additionally, overexpression of cyclin-dependent kinase, known to hyperphosphorylate the hGR in vitro, resulted in a decrease in the extent of ligand-induced recruitment of the histone acetyltransferase co-activator, p300 to the hGR bound on a MMTV-luciferase promoter in COS-1 and HCT116 cells (*2*). On the other hand, phosphorylation of the expressed hGR at S404, by glycogen synthase kinase 3β (GSK3β), was shown to be required for the recruitment of CBP/p300 (*29*). Studies on the ER demonstrated that the change in phosphorylation status at one specific serine residue determines whether the co-activator SRC-3 or the co-repressor, stromelysin-1 platelet-derived growth factor-responsive element-binding protein, is recruited (*18*, *31*−*33*).

Taken together, these studies suggest that the mechanisms by which agonist-induced phosphorylation of the GR modulates GR-mediated transcription may occur at multiple levels and be glucocorticoid dose-dependent, as well as promoter- and cell-specific. We have previously shown a direct correlation between efficacy for transactivation via the hGR and binding of the hGR to co-activators, in particular to GRIP-1 (*34*). Thus, a role for GR phosphorylation in the determination of GRIP-1 recruitment could be a mechanism whereby GR phosphorylation modulates transcription efficacy. However, whether phosphorylation of the hGR at S211, S226, and/or S203 affects co-activator recruitment has not previously been investigated. In this study, we investigated in COS-1 cells the role of hGR phosphorylation at S211, S226, and S203 in GR-mediated transactivation efficacy on the synthetic MMTV and endogenous GILZ GREs, as well as on GR protein degradation, nuclear localization, and GRIP-1 recruitment in vitro and in intact cells.

MATERIALS AND METHODS

Cell Lines and Inducing Compounds. Monkey kidney fibroblast cells (COS-1 and COS-7) were purchased from American Type Culture Collection. COS cells were cultured in high-glucose (1 g/mL) Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (Delta Bioproducts), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco Invitrogen, Paisley, U.K.) at 37 °C in a 5% CO_2 incubator. dex was purchased from Sigma-Aldrich and was made up in a 10 mM stock solution in ethanol and added to the cells such that the final concentration of ethanol was less than 0.1%. Z-Leu-Leu-Leu-al (MG132), α-amanitin, and cycloheximide (CHX) were purchased from Sigma-Aldrich.

Plasmids. The pTAT-GRE-E1b-luc plasmid or pTAT-GRE (E1b promoter; two copies of rat TAT-GRE) plasmid was a gift from G. Jenster at Erasmus University of Rotterdam (Rotterdam, The Netherlands) (*35*). The HA-tagged human GR pCMV (pHA-hGR) and mutants S211A pCMV (pHA-S211A) and S226A pCMV (pHA-S226A) were gifts from M. J. Garabedian (New York University, New York, NY), and the pCMV-β-gal plasmid (pβgal) was a gift from G. Haegeman (University of Gent, Gent, Belgium). The pRS human GR (pRS-hGR) and the pRS S203/211/226A (pRS-3A) mutant were gifts from T. Kino (National Institutes of Health, Bethesda, MD). HA-GRIP was a gift from M. R. Stallcup (University of Southern California, Los Angeles, CA). The MMTV-luciferase reporter (pMMTV), which contains four GREs, was a gift from G. L. Hager (National Cancer Institute, Bethesda, MD). The pTAT1-luc [containing a single TAT GRE linked upstream of the TATA box-containing Adh promoter (−33 to +53)] (*36*) and the pGREtk-luc constructs [containing two GREs linked upstream of the thymidine kinase promoter (−105 to +52), which contains two Sp1 sites and one CCAAT box, but no TATA box] were kind gifts from D. Pearce (University of California, San Francisco, CA) and S. Okret (Karolinska Institutet, Stockholm, Sweden), respectively.

Luciferase Reporter Assays. For transactivation assays, COS-1 cells were seeded into 10 cm dishes (NUNC, AEC Amersham) at a density of 1.5×10^6 cells per dish. The next day the cells were transfected with 10 μg of pHA-hGR, pRS-hGR, or pRS-3A and 3.75 μg og pMMTV-luc, pTAT1-luc, or pGREtk-luc construct, as well as pβgal, using FuGENE 6 (Roche). On day 3, cells were trypsinized and replated into 24-well dishes at a density of 7×10^4 cells per well. For the α-amanitin and MG132 studies, the cells were pretreated with increasing amounts of α-amanitin for 24 h or increasing amounts of MG132 for 4 h. Thereafter, the cells were stimulated with increasing amounts of dex for 16 h. The cells were then washed twice with PBS and lysed in 50 μL of reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the lysates was measured using the Luciferase Assay System (Promega) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase activity was normalized to protein content per well as determined by the Bradford assay or by β-galactosidase activity.

Western Blotting. COS-1 cells were seeded into 12-well plates (NUNC, AEC Amersham) at a density of 2×10^5 cells per well and transfected with 1 μg of pHA-hGR using Fugene transfection reagent (Roche). After 48 h, the cells were washed twice with PBS and then incubated for 1 h with serum free medium containing 100 nM dex. Subsequently, cells were washed with PBS and lysed in 50 μL of 2× SDS sample buffer (*37*), before equal amounts of protein were loaded on an 8% SDS−polyacrylamide gel. The proteins were blotted onto a Hybond-ECL nitrocellulose membrane (Amersham) using the Mini Protean III blotting system (Bio-Rad) for 1 h at 180 mA and room temperature (RT). Western blot analysis was performed according to standard procedures. Membranes were blocked in 4% ECL blocking buffer [4% (w/v) ECL Advance blocking powder (Amersham), 1× TBS (*37*), and 0.1% (v/v) Tween] for 1 h at RT, followed by incubation with anti-P-S211 or anti-P-S226 antibodies (1:10000) (from M. J. Garabedian) in 4% ECL blocking buffer overnight at 4 °C. Thereafter, membranes were washed with TBST for 1×15 and 2×5 min and incubated with anti-rabbit HRP antibody (1:10000) (Amersham) in buffer containing 5% milk powder [5% (w/v) milk powder, 1× TBS (*37*), and 0.1% (v/v) Tween] for 1 h at RT. Thereafter, the membranes were washed as described above, and detection was performed using ECL (Amersham). Quantification of total GR levels was performed on the same blot after the membrane had been stripped as previously described (*28*) and reprobed with an anti-GR antibody (H300, Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary antibody (anti-rabbit HRP at 1:10000) and visualization as described above. Bands on the X-ray film were quantified using Alpha Ease FluorChem.

GR Turnover. COS-1 cells were plated into 10 cm dishes (NUNC, AEC Amersham) at a density of 2×10^6 cells per dish and transfected with 10 μg of pRS-hGR or pRS-3A. The following day the cells were trypsinized and plated into six-well plates at a density of 4×10^5 cells per dish. After 24 h, the cells were serum-starved and pretreated with 1 μM cycloheximide for 1 h, before being stimulated with 10 μM test compound for various times. At the appropriate time, the cells were harvested and Western blot analysis was performed as described above, using primary antibodies to total GR (1:4000) (H300, Santa Cruz Biotechnology), β-actin (1:1500) (Cell Signaling), or GAPDH (1:1000) (Cell Signaling), as a loading control, and anti-rabbit HRP (1:10000) (Amersham) as the secondary antibody.

Co-Immunoprecipitation. COS-1 cells were seeded into 10 cm dishes (NUNC, AEC Amersham) at a density of 1.5×10^6 cells per dish. After a 24 h incubation, the cells were transfected with 3 μg of pHA-GRIP-1 and 3 μg of either pRS-hGR, pRS-3A, or pcDNA3.1 (empty vector) using FuGENE 6 (Roche) according to the manufacturer's instructions. The following day the cells were treated with 1 μM dex or vehicle for 1 h, washed once with PBS, and lysed with 500 μL of Cytobuster (Novagene) containing protease inhibitors [one complete mini protease inhibitor cocktail tablet per 10 mL (Roche)]. After removal of 9 μL of supernatant for inputs, 1.6 μg of anti-GR (H300, Santa Cruz Biotechnology) antibody was added to the remaining supernatant and incubated at 4 °C with overnight rotation. The following day the complexes were precipitated by addition of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 2 h at 4 °C while being rotated, followed by two washes with PBS. The GR-bound proteins in the pellet were released from the beads by being boiled in 20 μL of 2× SDS sample treatment buffer (*37*). The samples (18 μL) were separated via 6% SDS−PAGE, and Western blot analysis was performed using anti-GR (1:4000) (H300, Santa Cruz Biotechnology) and anti-HA (1:1000) (12CA5, Roche) primary antibodies or anti-rabbit HRP (1:10000) and anti-mouse HRP (1:2000) (Amersham) secondary antibodies.

Avidin−Biotin−Complex−DNA (*ABCD*) *Assay*. The ABCD assay was performed as previously described (*34*) with some modifications. Cytosols were prepared from COS-7 cells transfected with either pRS-hGR, pRS-3A, pHA-GRIP-1, or pcDNA3.1 (empty vector). The cytosols containing GR or empty vector were prepared in TAPS buffer (*38*), whereas the cytosols containing pHA-GRIP-1 were prepared using Cytobuster (Novagen) according to the manufacturer's instructions. On day 1 of the ABCD assay, 20 μL of wild-type (wt) or mutant GR or TAPS "mock" cytosol was diluted with 80 μL of HEPES wash buffer (*37*) and stimulated with 10 μM dex for 2.5 h on ice before being heat activated for 30 min at 20 °C. Thereafter, the activated liganded GR complexes were incubated with annealed, double-stranded biotinylated oligonucleotides (Invitrogen) containing a single GRE (from the rat TAT gene) immobilized on 40 μL of a 50% slurry of streptavidin−agarose beads (Sigma-Aldrich), overnight at 4 °C. The GRIP-1 (111 μL) cytosols were diluted with 89 μL of HEPES wash buffer and added to the GR−GRE−bead complex, followed by incubation for 4 h at 4 °C. The beads were washed and pelleted, before the proteins bound to the pelleted beads were assessed by Western blotting as previously described (*38*) using anti-GR (1:4000) (H300, Santa Cruz Biotechnology) or anti-HA (1:1000) (12CA5, Roche) as the primary antibody and anti-rabbit HRP (1:10000) or anti-mouse HRP (1:2000) (Amersham), respectively, as the secondary antibody.

Chromatin Immunoprecipitation (*ChIP*) *Assay*. The ChIP assay was performed as previously described (*39*) with some modifications. Briefly, COS-1 cells were transiently transfected in 10 cm dishes with 5 μg of pHA-GRIP, 3.75 μg of pMMTV-luc, and 10 μg of either pRShGR, pRS-3A, or empty vector (pcDNA3.1) and treated with 100 nM dex for 1 h, before being cross-linked with 1% formaldehyde. The formaldehyde was quenched with glycine, whereafter the cells were washed and resuspended in nuclear lysis buffer. After sonication, the lysates were centrifuged to remove the cellular debris. An aliquot of the supernatants (30 μg of chromatin) was removed and used as input, while 50 μg of the chromatin was immunoprecipitated

overnight with 5 μg of either the anti-GR antibody (H300, Santa Cruz Biotechnology) or an anti-HA antibody (Y-11, Santa Cruz Biotechnology), as well as an anti-IgG antibody (Santa Cruz Biotechnology) as a negative control. After incubation with protein A/G beads (Santa Cruz Biotechnology) and extensive washing, the immunoprecipitated DNA was eluted from the beads using elution buffer. After the cross-links were reversed overnight, the samples were treated with proteinase K (Roche) and the DNA was purified using the Qiagen PCR purification kit. The purified DNA was subjected to quantitative real-time PCR, using specific primers for the GRE region (−219 to −47) of the MMTV-luc promoter (MMTV F, 5′-AAC CTT GCG GTT CCC AG-3′; MMTV R, 5′-GCA TTT ACA TAA GAT TTG G-3′) (*2*) or to the promoter of the endogenous monkey GILZ gene, spanning the equivalent of GREs 3−6 (*40*) (GILZ F, 5′-AGT TAA GCT CCT GAT TTA AGA AG-3′; GILZ R, 5′-CCC GAT CTC AGG ACA TTC-3′), based on homology among the human, chimp, and rhesus monkey GILZ promoter sequences.

Subcellular Fractionation. COS-1 cells were plated into six-well plates (NUNC, AEC Amersham) at a density of 3×10^5 cells per well. After a 24 h incubation, the cells were transfected with 1 μg of either pRS-hGR, pRS-3A, or pcDNA3.1 (empty vector) using FuGENE 6 (Roche) according to the manufacturer's instructions. The following day the cells were treated with 100 nM dex or vehicle for 1 h, washed once with PBS, resuspended in a low-ionic strength buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM DTT, and 0.05% NP40], and incubated on ice for 10 min. Thereafter, the nuclear pellet and cytoplasmic fractions were separated by centrifugation. Equal fractions of the total cytoplasmic and nuclear fractions were separated via 10% SDS−PAGE. Western blotting was performed using anti-GR (H300, Santa Cruz Biotechnology), anti-GAPDH (14C10, Cell Signaling), and anti-histone H3 (ab1791, Abcam) antibodies.

Quantitative Real-Time PCR. COS-1 cells were plated into 12-well plates (NUNC, AEC Amersham) at a density of 1.5×10^5 cells per well. After a 24 h incubation, the cells were transfected with 0.5 μg of either pRS-hGR, pRS-3A, or pcDNA3.1 (empty vector) using FuGENE 6 (Roche) according to the manufacturer's instructions. The following day the cells were serum-starved for 2 h, before being treated with 100 nM dex or vehicle for an additional 2 h. Total RNA from COS-1 cells was isolated with Trizol reagent (Sigma-Aldrich) according to the manufacturer's instructions, and 0.5 μg of RNA was reverse-transcribed using the Transcriptor First Strand cDNA Kit (Roche) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SensiMix dT kit (Quantace) and primers for GILZ (QuantiTech Primer Assay 249900, Qiagen) and GAPDH forward and reverse primers [5′-TGA ACG GGA AGC TCA CTG G-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (*41*), respectively] under the following conditions: 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. Melting curve analysis and gel electrophoresis were performed to confirm the generated amplicon in each sample. Relative GILZ transcript levels were calculated with the "Fit Points" method described by Pfaffl et al. (*42*) and were normalized to relative GAPDH transcript levels.

Statistical Analysis. Statistical analyses were conducted using GraphPad Prism, using one-way analysis of variance with Bonferroni (which compares all treatments to each other) and/or Dunnett (which compares all values to a set control) post tests. Statistical significance of differences is denoted by one ($P < 0.05$), two ($P < 0.01$), or three asterisks ($P < 0.001$). The letters a, b, c, etc., are also used to denote statistically significant differences, where all those values that differ significantly from others are assigned a different letter.

RESULTS

The hGR Displays Rapid Agonist-Induced Phosphorylation at S211 and S226. Stimulation with dex resulted in hGR hyperphosphorylation at both S211 and S226 (Figure 1A,B) in COS-1 cells, consistent with previous results of others in different cells (*23*, *29*). The level of agonist-induced phosphorylation at S211 increases rapidly over time, reaching a maximum at ~60 min, whereas agonist-induced phosphorylation at S226 is less pronounced and does not change significantly over time (Figure 1C,D). The specificity of the phosphoserine antibodies (*23*, *24*) was confirmed using the GR S211A, S226A, and 3A phosphorylation mutants (Figures 10 and 12A,B of the Supporting Information).

Proteasomal Degradation and RNA Polymerase II Activity Are Differentially Required for Agonist-Mediated hGR Phosphorylation at S226 and S211. To investigate in more detail the relationship between hGR phosphorylation and transcription, as well as hGR phosphorylation and degradation, α-amanitin and MG132 were used to inhibit transcription and proteasomal degradation, respectively. Suitable concentrations of α-amanitin and MG132 for inhibition of transcription and degradation were first established (Figure 11A−D of the Supporting Information). To test the effect of α-amanitin and MG132 on hGR phosphorylation, cells were treated with 2.5 μg/mL α-amanitin or 10 μM MG132 before being stimulated with dex. Equal amounts of cell extracts were separated via SDS−PAGE, and Western blotting was performed with probing for either anti-phospho-S211 (Figure 2A) or anti-phospho-S226 (Figure 2B). The results show that inhibiting transcription has no statistically significant effect on S211 phosphorylation, whereas blocking hGR degradation slightly decreases the extent of dex-mediated S211 GR phosphorylation (Figure 2C). However, this decrease is not statistically significant. Interestingly, the amount of basal S226 phosphorylation increased when transcription was inhibited and increased even more when GR degradation was blocked (Figure 2B). Additionally, the amount of dex-dependent S226 phosphorylation decreased when either transcription or GR degradation was inhibited (Figure 2D). These results suggest that proteasomal degradation is required for agonist-mediated hGR phosphorylation at S226 and possibly at S211, while transcription is required for agonist-mediated hGR phosphorylation at S226 but not at S211.

hGR Phosphorylation at S203, S211, and/or S226 Is Required for Transactivation on an MMTV Promoter. To further investigate the role of GR phosphorylation, experiments were conducted with a triple hGR phosphorylation mutant (3A) containing alanine residues in place of S226, S211, and S203. This strategy was chosen rather than using single site mutations, since it has previously been suggested that the sites can compensate for each other (*43*). The S203 mutation was included since earlier reports in the literature indicated that the level of phosphorylation at S203 is weakly increased upon dex stimulation (*24*). The absence of phosphorylation at S211 and S226 in this mutant construct was confirmed using GR phosphoserine-specific antibodies (Figure 12A,B of the Supporting Information).

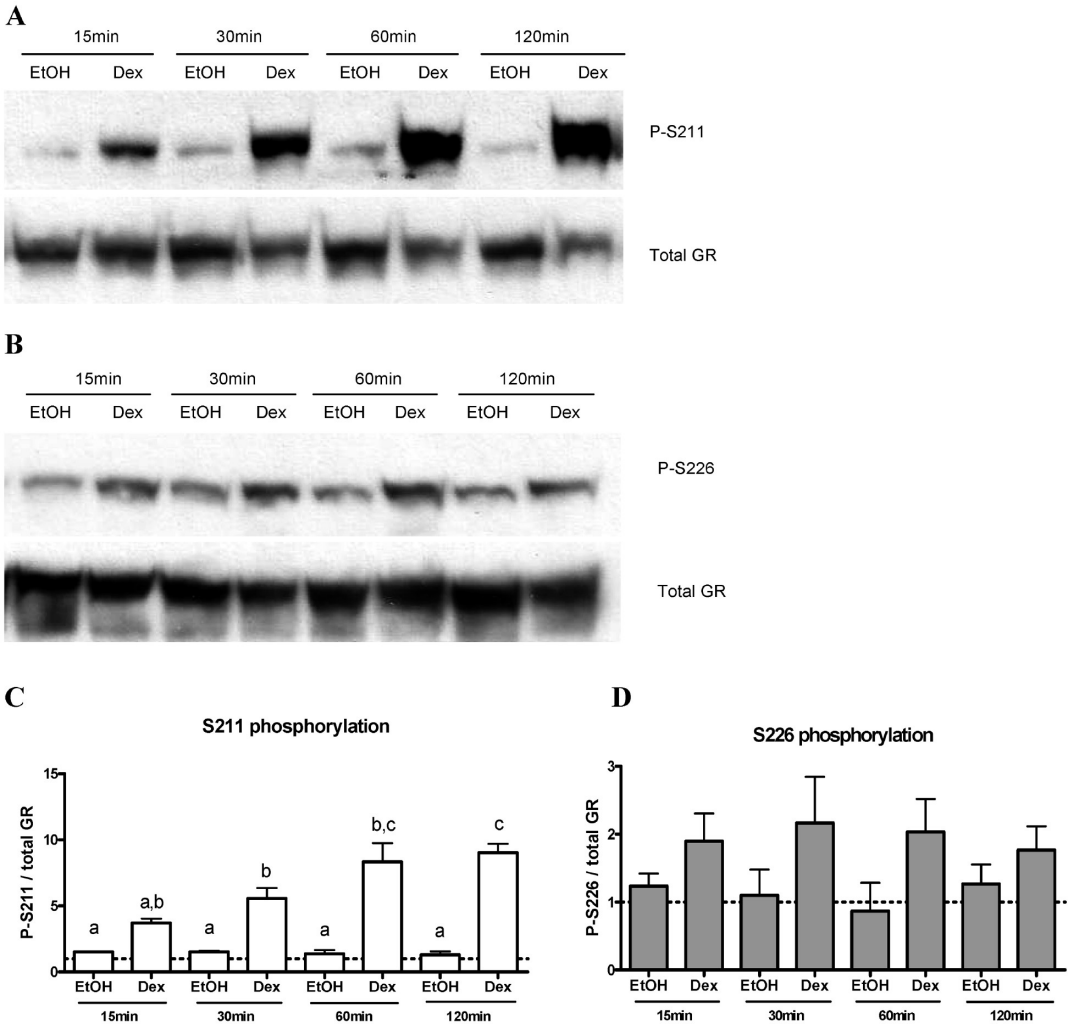

FIGURE 1: hGR displays rapid agonist-induced phosphorylation at S211 and S226. COS-1 cells transiently transfected with pHA-hGR were treated with vehicle (EtOH) or 100 nM dex, and cell lysates were harvested at different times as indicated. Western blot analysis was performed using either an anti-phospho-S211 (P-S211) (A) or an anti-phospho-S226 (P-S211) (B) GR-specific antibody. Thereafter, the membrane was stripped and reprobed with anti-GR antibody for total GR. After quantification, the amount of phosphorylated GR in COS-1 cells was normalized to the total amount of GR and plotted as fold induction. The Western blots are representative figures (A and B), and graphs (C and D) show the pooled results from four independent experiments. In panel C, letters a, b, and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$).

The significance of mutating all three phosphorylation sites on transactivational efficacy was examined on three different GRE-containing synthetic reporter genes. COS-1 cells transfected with pβgal and pMMTV, pGREtk-luc, or pTAT1-luc, containing four, two, or one GRE, respectively, as well as pRS-hGR or pRS-3A expression constructs, were stimulated with dex for 24 h. As one can see in Figure 3A, the phosphorylation mutant exhibited a small but not statistically significant dex induction on both the pMMTV and pTAT1-luc constructs. On the other hand, on the pGREtk-luc construct, the phosphorylation mutant had the same maximal efficacy for transactivation as the wt receptor. Western blotting confirmed that the wt and triple mutant receptors were expressed at similar levels in all these experiments (Figure 3B). These results show that in COS-1 cells, phosphorylation at one or more S residues (from S203, S211, and S226) of the GR is required for maximal transactivation on the MMTV-luc and TAT1-luc promoters, but not on the TK-luc promoter.

Phosphorylation at S203, S211, and/or S226 Is Not Required for hGR Degradation. The effect of the simultaneous mutation of S226, S211, and S203 on GR half-life was examined next. In the unliganded form, the GR is degraded slowly over time, and there is no difference between the half-life of wt and triple mutant for the unliganded GR (Figure 4A,B). Upon dex stimulation, the GR is degraded more rapidly, resulting in a shorter half-life for both wt and the triple phosphorylation mutant (Figure 4A,C). No significant difference was observed between the half-life of wt and that of the triple phosphorylation mutant for the liganded GR. These results show that phosphorylation at one or more S residues (from S203, S211, and S226) is not required for degradation of the liganded or unliganded hGR in COS-1 cells.

Phosphorylation of the hGR at S203, S211, and/or S226 Does Not Influence GR Nuclear Translocation. To determine whether phosphorylation of the hGR plays a role in GR nuclear translocation, COS-1 cells, transiently transfected with pRS-hGR or pRS-3A, were stimulated with 100 nM dex or vehicle for 1 h. Thereafter, cytoplasmic and nuclear fractions were prepared and separated via SDS−PAGE, followed by Western blotting with anti-GR, anti-GAPDH, and anti-histone H3 antibodies. wt and 3A GR were expressed similarly in the

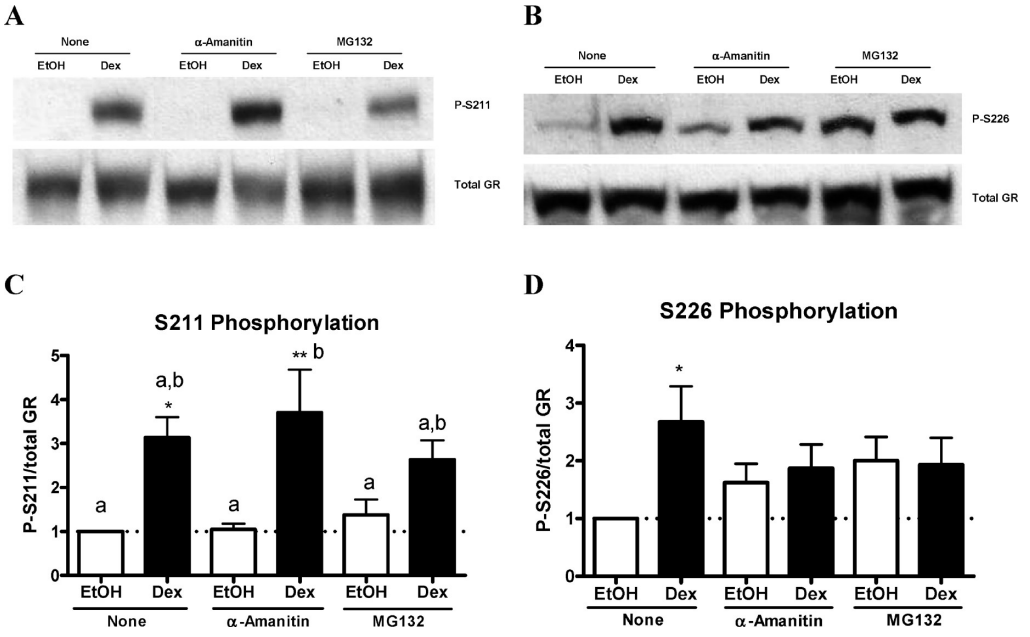

FIGURE 2: Proteasomal degradation and RNA polymerase II activity are differentially required for agonist-mediated hGR phosphorylation at S226 and S211. COS-1 cells transiently transfected with pHA-hGR were pretreated with either 10 μM MG132 for 4 h or 2.5 μg/mL α-amanitin for 24 h before being stimulated with vehicle (EtOH) or 100 nM dex for an additional 1 h. Equal amounts of samples were loaded onto an 8% SDS−PAGE gel, and Western blotting was performed, probing with either an anti-P-S211 (A) or anti-P-S226 antibody (B). After development, the blots were stripped and reprobed for total GR. The amount of phosphorylated S211 and S226 was normalized to total GR and plotted as the average of four independent experiments ± SEM (C and D), while the Western blots in panels A and B show one representative result. In panels C and D, statistical significance of differences, when using the Dunnett post test, is denoted by one ($P < 0.05$) or two asterisks ($P < 0.01$), where all values are compared to that of the wild-type vehicle. In panel C, the letters a and b are used to denote statistically significant differences when using the Bonferroni post test, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$).

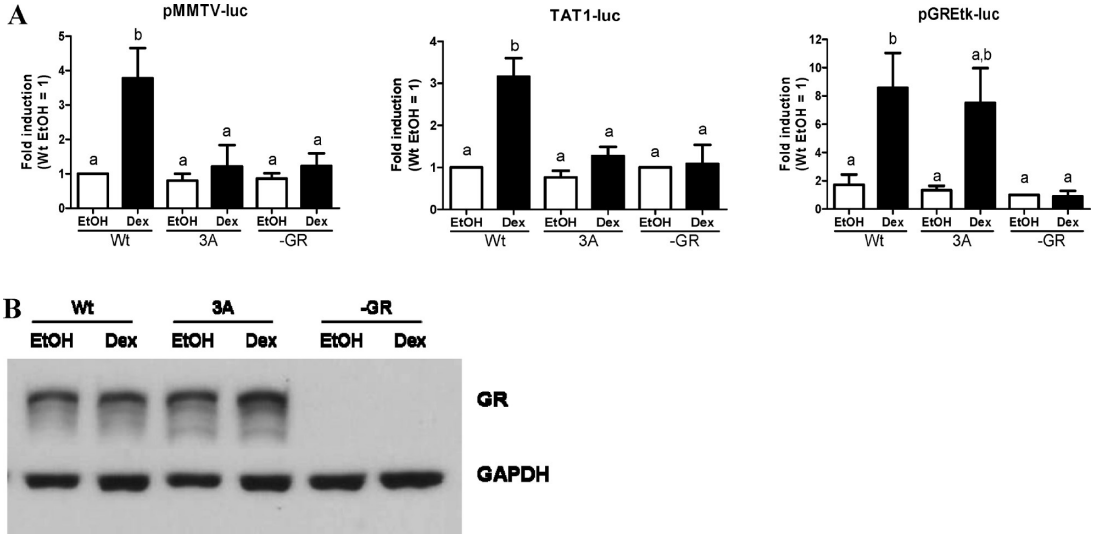

FIGURE 3: hGR phosphorylation at S203, S211, and/or S226 is required for transactivation on an MMTV and a TAT1-luc promoter but not on a TK-luc promoter. COS-1 cells transiently transfected with pβgal, pRS-hGR, or pRS-3A, as well as pMMTV, pTAT1-luc, or pGREtk-luc (A), were treated with vehicle (EtOH) or 100 nM dex for 24 h. Luciferase activity in the cell lysates was normalized to βgal activity per well. After the luciferase and βgal assays were performed, equal amounts of sample were loaded onto an 8% SDS−PAGE gel and Western blotting was performed, probing with either an anti-GR or an anti-GAPDH antibody as a loading control (B). The histograms (A) show pooled results from at least two independent experiments, performed in parallel, where each condition was performed in triplicate, and average values are plotted as means ± SEM, expressed as fold induction relative to vehicle (EtOH) with the wt value set to 1. The Western blot (B) shows one representative blot. The letters a, b, and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$).

input (Figure 5A). The cytoplasmic GAPDH and nuclear H3 Western blots show that pure fractions were obtained (Figure 5B). In the absence of ligand, the hGR is predominantly cytoplasmic, whereas stimulation with dex results in GR nuclear translocation with ~50% of the hGR located in the nuclei (Figure 5C). Similar results with ~50% of the hGR located in the nuclei were also obtained under the same experimental conditions for endogenous hGR in U20S cells, which express

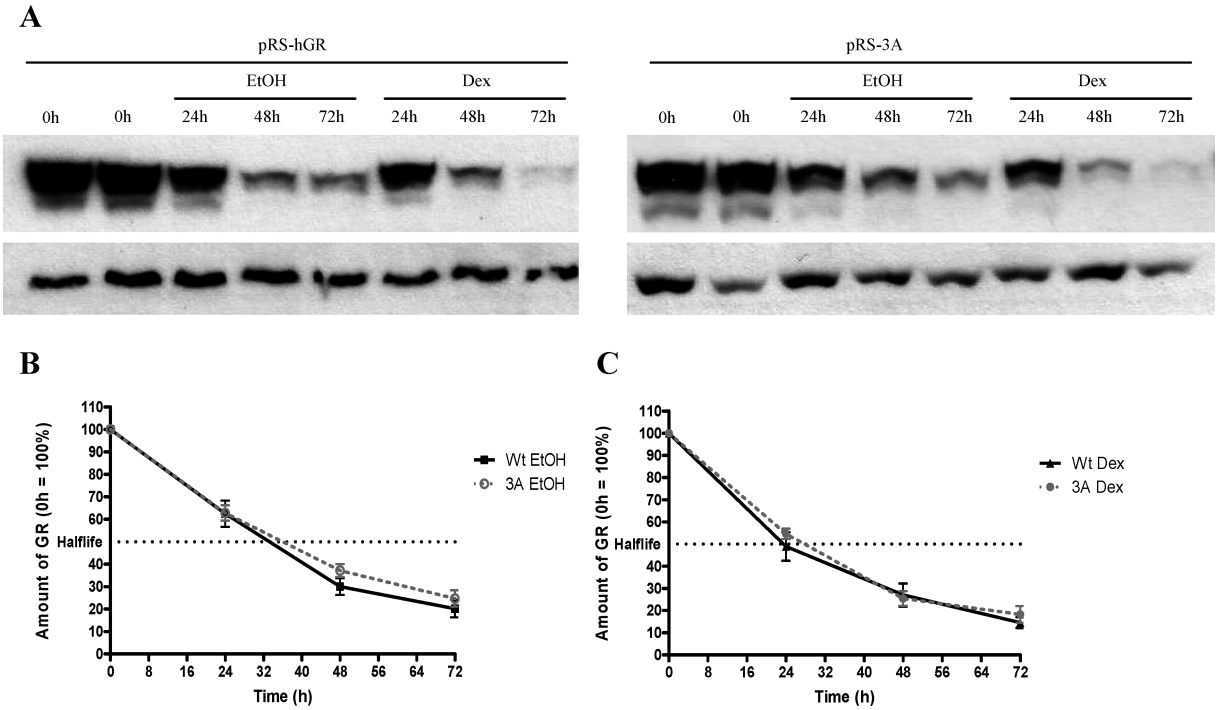

FIGURE 4: Phosphorylation at S203, S211, and/or S226 is not required for hGR degradation. COS-1 cells were plated in 10 cm dishes at a density of 2×10^6 cells per dish and transfected with either pRS-hGR or pRS-3A. After a 24 h incubation, cells were replated into six-well plates at a density of 4×10^5 cells per well. The following day the cells were pretreated with 1 μM cycloheximide for 1 h and then treated with vehicle (EtOH) or 10 μM dex. At the appropriate time, whole cell extracts were prepared and equal amounts of protein were analyzed by Western blotting and probed with anti-hGR and anti-β-actin antibody. A single representative Western blot is shown for each GR construct. (A). After quantification and normalization to β-actin, the amount of GR present at 0 h was set to 100%, and the amount of GR present at the other time points was calculated as a percentage thereof (B and C). Graphs show the average of four independent experiments, with values plotted as means ± SEM.

very low levels of the endogenous GR (data not shown). Furthermore, no difference between wt and 3A GR subcellular localization in the absence or presence of dex was detected. These results show that phosphorylation at S203, S211, and/or S226 does not influence the extent of hGR nuclear translocation, suggesting that the decrease in transcriptional activity on the MMTV and TAT1-luc promoters is not due to deficient nuclear translocation of the triple phosphorylation mutant.

Phosphorylation of the hGR at S203, S211, and/or S226 Is Required for GRIP-1 Binding, but Not for DNA Binding in Vitro. To investigate whether mutation of the three serine residues results in a loss of GR DNA binding per se, we performed an in vitro DNA pull-down ABCD assay using biotinylated GREs (*34*). Both wt and 3A GR were expressed at similar levels (Figure 6A), and the same GRIP-1 cytosols were used for wt and GR mutant pull-down assays. These results show that the same amount of GR was bound to the biotinylated GRE for wt and 3A GR (Figure 6B and Figure 14A of the Supporting Information). However, stimulation with dex resulted in recruitment of more GRIP-1 for wt GR than for the 3A GR mutant. Normalization and quantification revealed that dex stimulation led to an ~4.5-fold increase in the level of GRIP-1 binding for wt GR, while no significant GRIP-1 binding occurred for the 3A GR (Figure 6C and Figure 14B of the Supporting Information). Additionally, no GRIP-1 binding was detected in the absence of ligand or in the absence of expressed GR, showing a requirement for the GR for association of GRIP-1 with the DNA. These results show that phosphorylation of the GR at one of more S residues (from S203, S211, and S226) is required for GRIP-1 binding, but not for DNA binding in vitro.

hGR Phosphorylation at S203, S211, and/or S226 Is Required for Binding of Co-Activator GRIP-1 in Vitro. To investigate the role of GR phosphorylation at S203, S211, and/or S226 in cofactor recruitment, precipitation with the anti-GR antibody was used to investigate the interaction between GRIP-1 and the triple phosphorylation hGR mutant. HA-GRIP-1 was expressed at similar levels in the lysates, and expression of the wt GR was similar to the expression of the triple phosphorylation mutant (Figure 7A). Additionally, no endogenous GR could be detected (Figure 7A). In the absence of hormone, no GRIP-1 was co-immunoprecipitated with the GR, while dex stimulation led to the association of high detectable levels of GRIP-1 (Figure 7B). Interestingly, with the triple phosphorylation mutant, no GRIP-1 was co-immunoprecipitated with the GR in the absence or presence of dex, while the levels of GR immunoprecipitated for wt and triple phosphorylation mutant were similar (Figure 13A of the Supporting Information). After quantification and normalization, it was found that the wt GR associates with GRIP-1 in a ligand-dependent manner (~4-fold increase), whereas there is very little GRIP-1 association with the triple phosphorylation mutant (~1.6-fold) (Figure 7C and Figure 13B of the Supporting Information). The lack of GRIP-1 binding in the absence of expressed GR demonstrates that the presence of the hGR is required for GRIP-1 co-immunoprecipitation. Immunoprecipitation with a nonspecific IgG antibody did not result in any detectable GR or HA-GRIP-1 (Figure 7B, right panel). These results show that phosphorylation of the hGR at one or more S residues (from S203, S211, and/or S226) is required for the interaction of the GR with GRIP-1.

Phosphorylation of the hGR at S203, S211, and/or S226 Is Required for GRIP-1 and Maximal Recruitment of the GR to the MMTV Promoter in Intact Cells. Having shown that phosphorylation of the GR at S203, S211, and/or S226 is required for GRIP-1 binding in vitro and transactivation via the

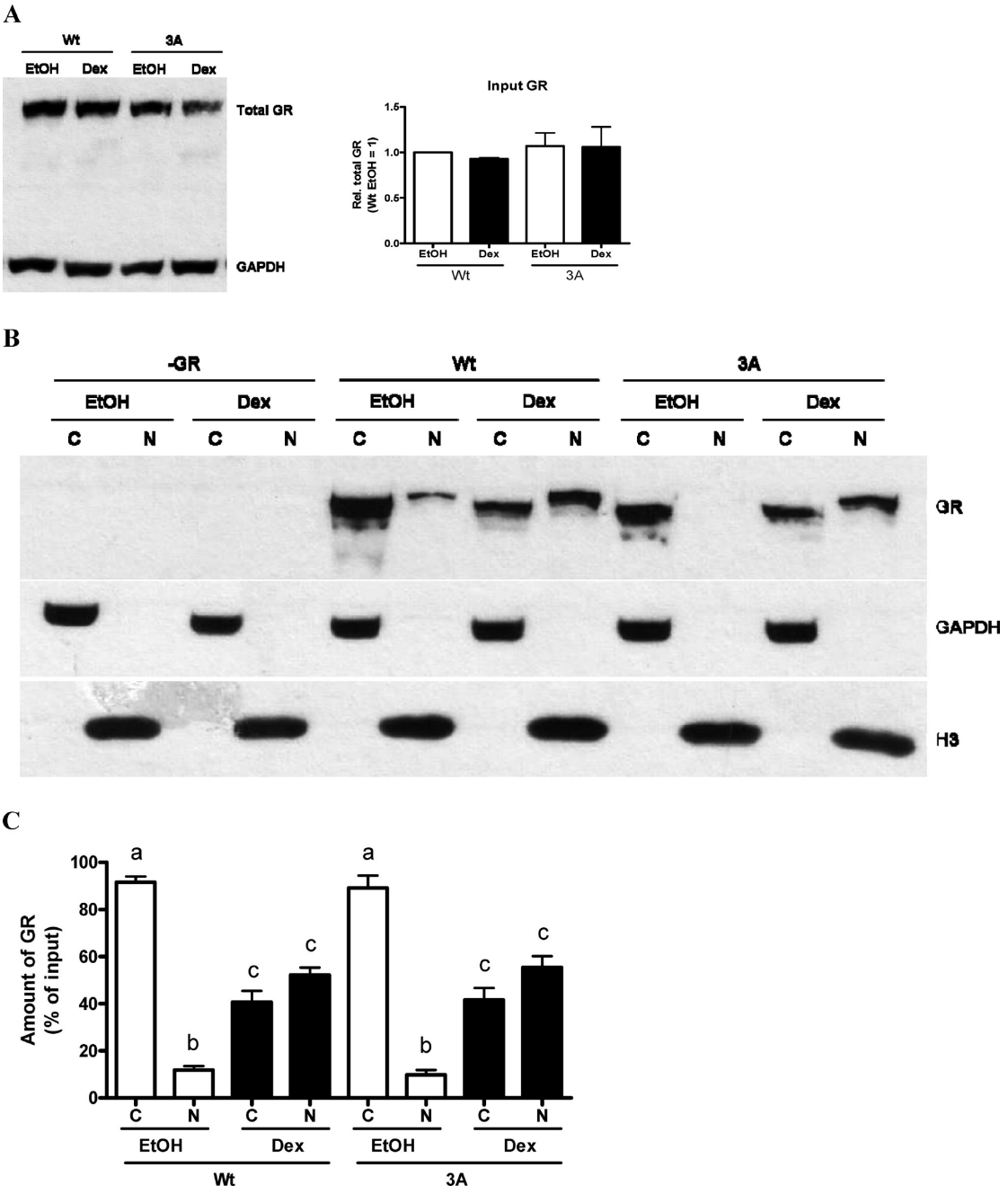

FIGURE 5: Phosphorylation of the hGR at S203, S211, and/or S226 does not influence GR nuclear translocation. COS-1 cells transiently transfected with either pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR) were treated with vehicle (EtOH) or 100 nM dex for 1 h. Western blotting with an anti-GR antibody was performed on the input samples (A), before cytoplasmic and nuclear fractions were prepared. Thereafter, equal amounts of the fractions were separated via SDS−PAGE, and Western blotting with anti-GR (GR), anti-GAPDH (GAPDH), and anti-histone H3 (H3) was performed (B). After quantification, the amount of GR in the input was set to 100% and the amount of GR in the cytoplasmic and nuclear fractions was calculated as a percentage of input (C). The blots (A and B) are single representatives from four independent experiments, and the graph in C is pooled data from four independent experiments, with values plotted as averages ± SEM. In panel C, the letters a, b, and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$). C, cytoplasmic fractions; N, nuclear fractions.

pMMTV reporter construct, we next determined whether GR phosphorylation is required for recruitment of GRIP-1 to the MMTV promoter in intact cells. COS-1 cells transiently transfected with pHA-GRIP-1 and pMMTV, as well as either pRS-hGR or pRS-3A, were stimulated with dex for 1 h, and a ChIP assay was performed, immunoprecipitating with an anti-HA antibody. Quantitative real-time PCR of the immunoprecipitated DNA revealed that the dex-mediated increase in the extent of recruitment of GRIP-1 to the wt GR is 3-fold, while 3A did not show any statistically significant dex-mediated increase in the extent of GRIP-1 recruitment (Figure 8A). Control experiments conducted in the absence of the expressed GR (Figure 8A) reveal that the immunoprecipation is specific for the expressed GR. These results show that phosphorylation of the GR is needed for dex-mediated recruitment of GRIP-1 to the GREs of the MMTV promoter in intact cells.

To determine whether the triple phosphorylation GR mutant could still bind to the GRE, and whether the lack of GRIP-1

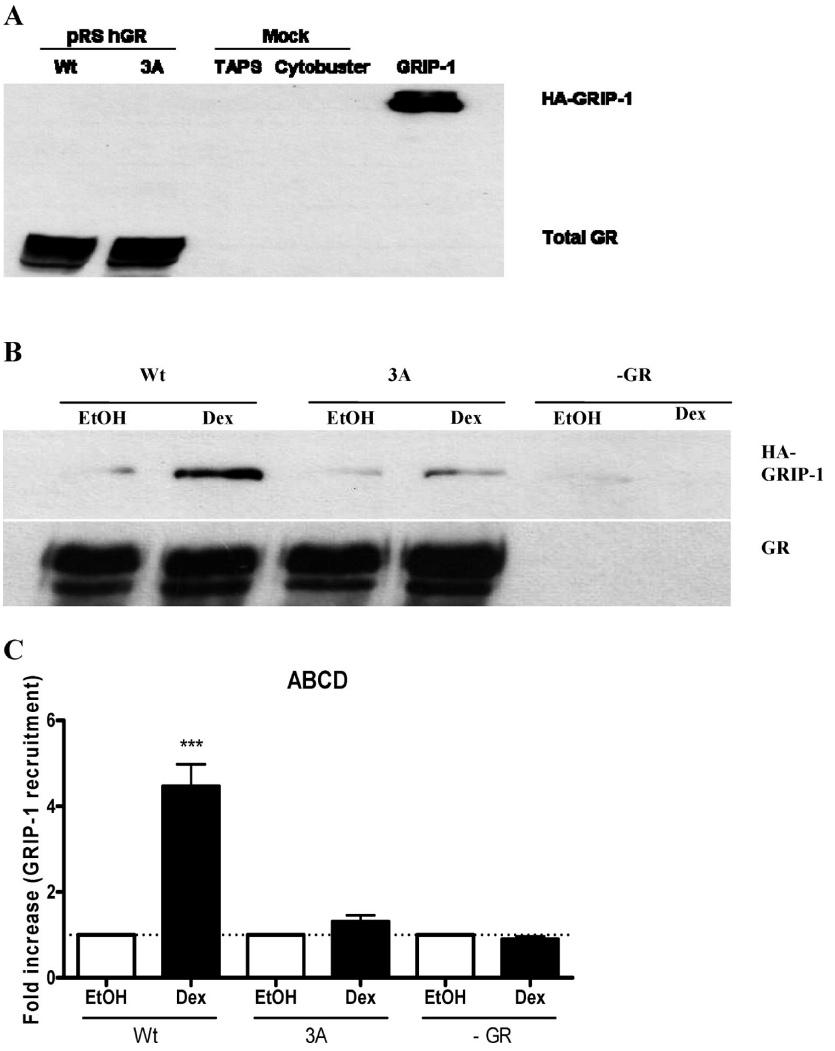

FIGURE 6: Phosphorylation of the hGR at S203, S211, and/or S226 is required for GRIP-1 binding, but not for DNA binding in vitro. COS-7 cytosols (20 μL) containing expressed pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR) were incubated with vehicle (EtOH) or 10 μM dex. Thereafter, the cytosols were incubated with biotinylated GRE oligonucleotides attached to streptavidin beads followed by incubation with COS-7 cytosols containing overexpressed HA-GRIP-1. Equal amounts of input cytosol (A) as well as DNA-bound proteins (B) were separated via SDS−PAGE and visualized by Western blotting probing with anti-HA and anti-GR antibodies. After quantification and normalization to GR levels, the amount of HA-GRIP-1 bound to the GR−DNA complex was plotted (C). The graph shows pooled results of three independent experiments, with averages thereof plotted as fold GRIP-1 bound, relative to the vehicle value set to 1. In panel C, statistical significance of differences is denoted by three asterisks ($P < 0.001$).

recruitment with the 3A mutant is due to less binding of the mutated GR to the GRE, a GR ChIP assay was performed. Quantitative real-time PCR of the immunoprecipitated DNA revealed that the extent of recruitment of the wt GR to the GRE increased ~8-fold upon dex stimulation, whereas the extent of recruitment of the triple phosphorylation mutant increased by only 2.8-fold (Figure 8B). This difference in DNA recruitment was not due to less GR present in the input, since the amount of wt GR in the sonicated chromatin was found to be similar to or slightly smaller than the amount of 3A GR, as determined by Western blotting with a GR antibody (Figure 8C). These results show that phosphorylation of the hGR at S203, S211, and/or S226 is required for GRIP-1 and maximal recruitment of the GR to the MMTV promoter in intact cells.

Phosphorylation of the hGR at S203, S211, and/or S226 Is Required for Maximal Transactivation Efficacy, as Well as GRIP-1, but Not Recruitment of the GR to the Endogenous GILZ Promoter. Having shown that phosphorylation at one or more S residues (from S203, S211, and S226) of the hGR is required for a maximal transcriptional response on the synthetic MMTV promoter as well as for recruitment of GRIP-1 to the MMTV promoter in intact cells, we sought to confirm this mechanism on an endogenous GRE-containing gene in the same cell system. Since our results showed that not all GRE-containing synthetic genes are sensitive to the phosphorylation status of the GR, and the MMTV gene is not expressed endogenously in COS-1 cells, we chose to investigate whether expression of the endogenous GILZ is sensitive to the phosphorylation status of the GR. COS-1 cells transiently transfected with pRS-GR or pRS-3A were stimulated with 100 nM dex or vehicle (EtOH) followed by RNA isolation and quantitative PCR analysis. The results show that GILZ mRNA levels are significantly upregulated in response to dex with the wt GR (Figure 9A). Similar to the results found on the MMTV and TAT1-luc constructs, dex stimulation resulted in a much reduced increase in GILZ mRNA levels with the phosphorylation mutant (Figure 9A).

Having shown that phosphorylation at one or more S residues (from S203, S211, and S226) is required for maximal efficacy for transactivation on the endogenous GILZ gene, we next

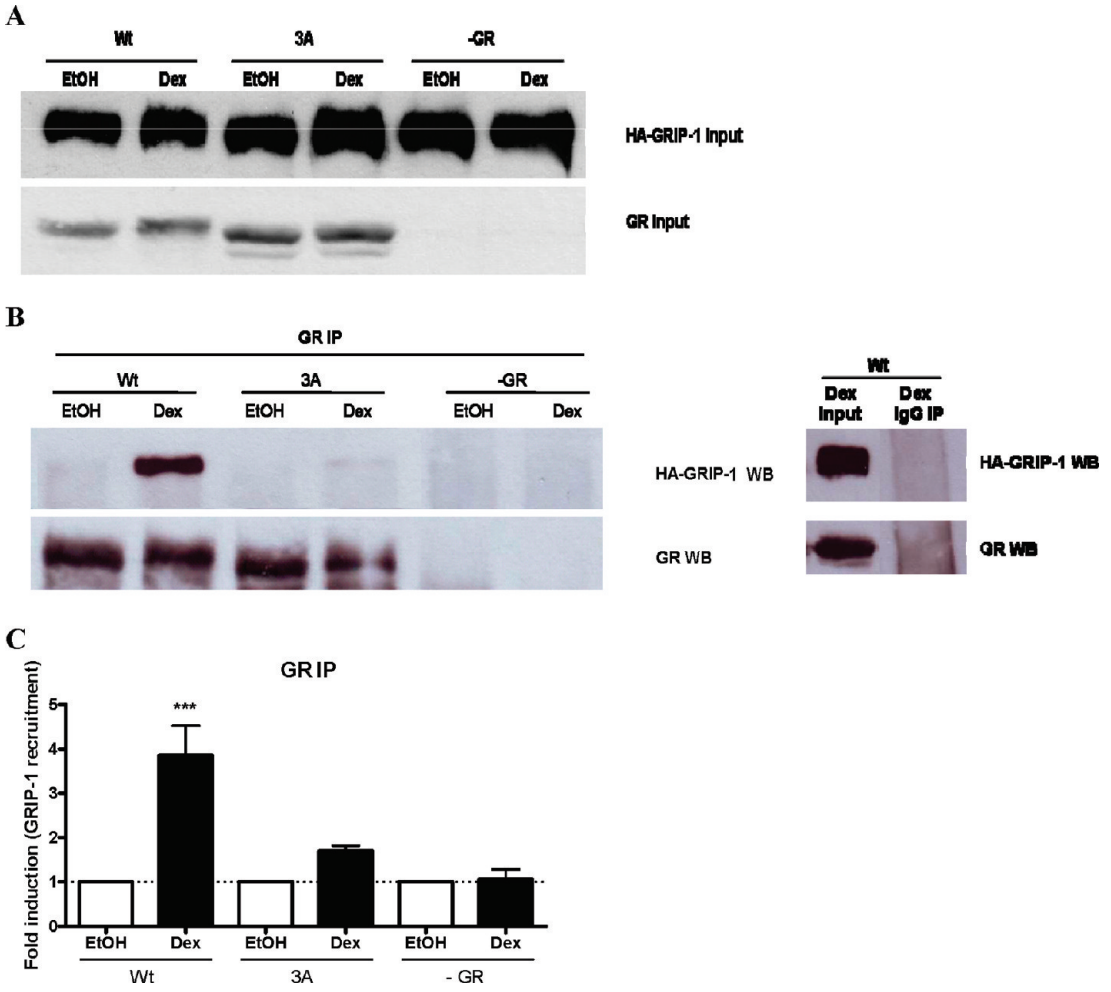

FIGURE 7: hGR phosphorylation at S203, S21, and/or S226 is required for binding of co-activator GRIP-1 in vitro. COS-1 cells transiently transfected with pHA-GRIP-1, as well as either pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR), were treated with vehicle (EtOH) or 100 nM dex for 1 h. After lysis, 1/50 of the cell lysate was subjected to SDS−PAGE and Western blotting and analyzed for input levels of the GR and HA-GRIP-1 (A). The remainder of the cell lysate was immunoprecipitated using an anti-GR antibody and protein A/G beads. Proteins bound to the complex were analyzed by Western blotting and probing for the GR and HA-GRIP-1 (B, left panel). The results of immunoprecipitation with a nonspecific IgG antibody are shown in the right panel of panel B. The amounts of HA-GRIP-1 and GR immunoprecipitated were normalized to their respective inputs. Thereafter, the relative dex-dependent GRIP-1 recruitment was plotted for each construct, where the value for vehicle (EtOH) was set to 1. Results show the averages ± SEM of two to four independent experiments (C). Statistical significance of differences is denoted by three asterisks ($P < 0.001$).

determined whether phosphorylation influences recruitment of the GR or GRIP-1 to the GILZ gene. Interestingly, on this endogenous gene, both wt and phosphorylation mutant GR were recruited to a similar extent in the presence of dex (Figure 9B). Similar to the results shown for the MMTV promoter, the wt GR recruited GRIP-1 in a statistically significant manner upon dex stimulation (3.6-fold), while the phosphorylation mutant (0.77-fold) failed to recruit GRIP-1 in a dex-dependent manner (Figure 9C). Western blotting showed that the wt GR is expressed at similar or slightly lower levels than the 3A mutant (Figure 9D).

DISCUSSION

The precise mechanism whereby GR phosphorylation modulates GR-mediated transcription remains unclear, despite its proposed involvement since 1990 (*19*). In our study, we investigated whether agonist-dependent hGR phosphorylation at one of more S residues (from S203, S211, and S226) affects GR-mediated transactivation, GR turnover, and GRIP-1 recruitment in COS-1 cells. We show that agonist binding results in hyperphosphorylation of the hGR at S211 and S226 in COS-1 cells, consistent with reports in the literature for the hGR in U2OS cells (*23*, *24*). Additionally, we show that this occurs rapidly, reaching a maximum ~1 h after agonist stimulation. The simultaneous mutation of three serine residues of the hGR [S203, S211, and S226 (3A mutant)] led to a significant and substantial reduction in the level of transactivation on the synthetic MMTV (four GREs) and TAT1-luc (one GRE) constructs, but not the GREtk-luc construct. This shows that phosphorylation of the hGR on one or more S residues (from S203, S211, and S226) is required for transactivation of the MMTV and TAT promoters, but not the GRE-tk promoter in COS-1 cells. Our results showing promoter-selective effects on transcription with GR phosphorylation mutants are consistent with other reports in the literature (*22*, *23*). Furthermore, they suggest that the sensitivity of promoters to the phosphorylation status of the GR does not correlate with the number of GREs and may thus be promoter-specific. Interestingly, while both the MMTV-luc and TAT1-luc constructs have promoters containing a TATA box (*45*, *36*), the GREtk-luc construct containing the thymidine kinase promoter does not; instead, it contains two Sp1 sites and a CCAAT box (*46*, *47*). Thus, it is possible that

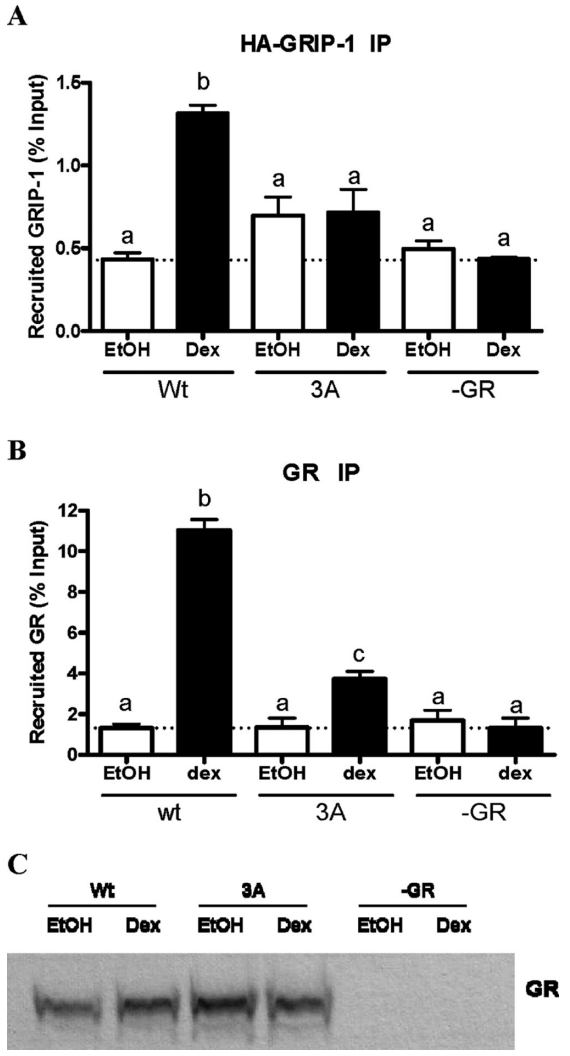

FIGURE 8: Phosphorylation of the hGR at S203, S211, and/or S226 is required for GRIP-1 and maximal recruitment of the GR to the MMTV promoter in intact cells. COS-1 cells transiently transfected with pHA-GRIP, pMMTV, and either pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR) were treated with vehicle (EtOH) or 100 nM dex for 1 h. ChIP was performed using either an anti-HA, anti-GR, or anti-IgG antibody. Quantitative PCR using PCR primers encompassing the GRE region of the MMTV-luc construct was performed on input chromatin, as well as the chromatin precipitated with an anti-HA antibody (A) or anti-GR antibody (B). Western blotting with an anti-GR antibody was performed on the input chromatin, and a representative result is shown (C). Results shown in panels A and B are from three independent experiments, with values plotted as averages ± SEM. Values are plotted as a percentage of input. In panels A and B, the letters a, b, and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$). Results with the IgG antibody showed no detectable signal on quantitative PCR and hence are not indicated on the histogram.

promoters lacking a TATA box are less sensitive to GR phosphorylation status at S203, S211, and S226, but further experiments would be required to test this hypothesis.

Having shown that agonist-induced phosphorylation modulates GR-mediated transactivation efficacy on the MMTV promoter, we further investigated the underlying mechanism. We show that proteasomal degradation appears to be required for maximal agonist-mediated hGR phosphorylation at S226 and possibly S211, while transcription appears to be required for maximal agonist-mediated hGR phosphorylation at S226 but not at S211. However, caution should be taken when interpreting this data, since we (data not shown) as well as others have shown that MG132 activates c-Jun N-terminal kinase (*44*), the kinase reported to phosphorylate the GR at S226, which could explain the increase in the basal level of S226 phosphorylation. Additionally, α-amanitin and MG132 are not specific for the GR and inhibit transcription and degradation of a variety of other proteins such as phosphatases, which could lead to indirect effects on GR phosphorylation. Nevertheless, taken together, these results would be consistent with a mechanism whereby agonist-mediated GR phosphorylation at S226 and possibly S211 is coupled to protein turnover. In addition, the results suggest that agonist-mediated phosphorylation at S211 occurs before GR-mediated transcription, consistent with a role for S211 phosphorylation in co-activator recruitment during transcription initiation.

One possible mechanism whereby GR phosphorylation modulates transactivation efficacy could be via mediating changes in GR turnover. Glucocorticoids have been shown to downregulate GR mRNA as well as GR protein levels (*17*, *26*). To determine whether hGR phosphorylation at S203, S211, and S226 plays a role in GR protein degradation in our system, we examined the effect of mutating the serine residues on hGR half-life. We show that in the absence of ligand, the hGR is slowly degraded and that agonist decreases the half-life of degradation. In addition, the combined mutation of all three sites, i.e., S211, S226, and S203, had no effect on the half-life of the unliganded or liganded hGR, suggesting that in COS-1 cells, phosphorylation at these three sites does not play a role in hGR protein turnover. This suggests that the mechanism whereby phosphorylation at S226, S211, and S203 modulates transactivation efficacy of the hGR in COS-1 cells does not involve modulation of hGR degradation. Our results with the unliganded hGR are in agreement with those of others for the unliganded mGR (*22*), while these authors found that a similar mGR mutation resulted in a loss of dex-induced increase in half-life, possibly due to different experimental conditions.

Whether GR phosphorylation affects GR nuclear translocation appears to be controversial (*22*, *24*). We show here that the wt and triple phosphorylation hGR mutant exhibit a similar degree of dex-mediated nuclear accumulation. Consistent with our results, Webster et al. showed that the combined mutation of the mouse GR residues equivalent to the human S203, S211, and S226 GR residues did not influence mGR nuclear import (*22*).

Having shown that the mechanism whereby GR phosphorylation inhibits transactivation on an MMTV promoter does not appear to be due to changes in GR turnover, DNA binding, or nuclear translocation, we investigated whether GR phosphorylation affects GRIP-1 co-activator recruitment. Steroid receptor phosphorylation-dependent recruitment of co-activators and co-repressors has been shown to play an important role in ER transcriptional activity. Phosphorylation at a single serine residue (S118) is required for ER interaction with the co-activator SRC-3 (*31*). Interestingly, the ER S118 residue is in the AF-1 domain, as are S203, S211, and S226 of the GR. Chen et al. proposed a model whereby phosphorylation of S211 influences transcriptional activity via modulating the interaction between the hGR and MED14, but their results indicate that hGR phosphorylation affects the interaction of the GR with additional factors in U20S cells (*23*). Our results using co-immunoprecipitation experiments in the absence and presence of a GRE with full-length GRIP-1 and hGR suggest that phosphorylation of the

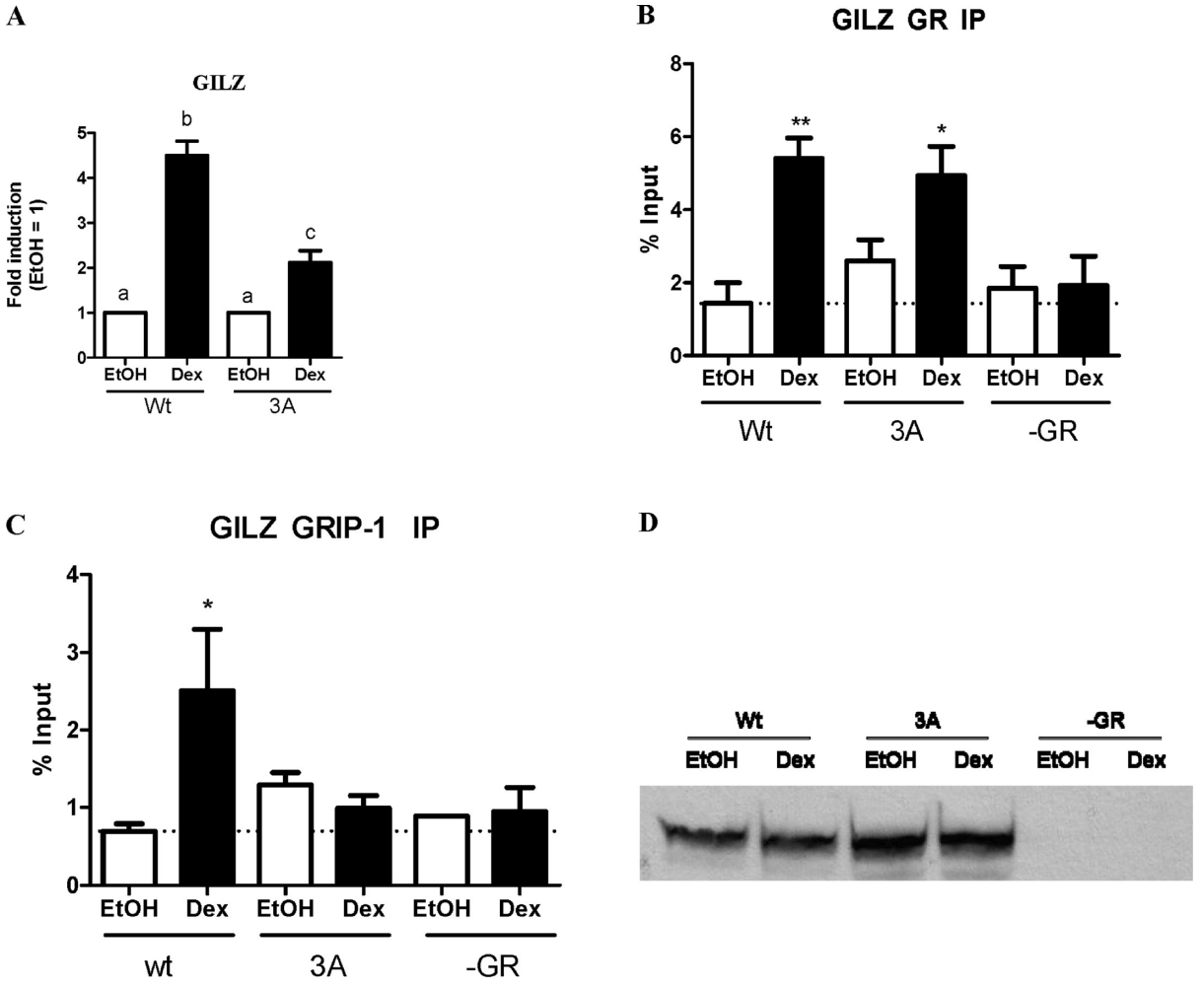

FIGURE 9: Phosphorylation of the hGR at S203, S211, and/or S226 is required for maximal transactivation efficacy, as well as GRIP-1, but not recruitment of the GR to the endogenous GILZ promoter. For the transactivation assay (A), COS-1 cells transiently transfected with either pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR) were serum-starved for 2 h before being treated with vehicle (EtOH) or 100 nM dex for an additional 2 h. Thereafter, total RNA was isolated and reverse-transcribed, and relative levels of GILZ transcripts were determined by quantitative real-time PCR. Fold changes in GILZ mRNA levels were normalized to GAPDH transcripts and were calculated relative to Dex-treated wt (Wt Dex = 100%) samples. The histogram shows pooled results from three independent experiments. For the ChIP assay (B−D), COS-1 cells transiently transfected with pHA-GRIP and either pRS-hGR (Wt), pRS-3A (3A), or empty vector (−GR) were treated with vehicle (EtOH) or 100 nM dex for 1 h. ChIP was performed using either anti-HA, anti-GR, or anti-IgG antibody. Quantitative PCR using PCR primers encompassing GREs 3−6 of the GILZ gene was performed on input chromatin, as well as the chromatin precipitated with anti-GR antibody (B) or anti-HA antibody (C). Western blotting with an anti-GR antibody was performed on the input chromatin, and the result from one representative experiment of three is shown in panel D. Results shown in panels B and C are pooled from three independent experiments, with values plotted as averages ± SEM. Values are plotted as a percentage of input DNA. In panel A, the letters a, b, and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$) while those having different letters are statistically significantly different from each other ($P < 0.05$). Statistical significance of differences in panels B and C is denoted by one ($P < 0.05$) and two asterisks ($P < 0.01$). Results with the IgG antibody showed no detectable signal on quantitative PCR and hence are not indicated on the histogram.

hGR at S226, S211, and S203 is required for GRIP-1 binding and transactivation of this promoter in intact cells. Indeed, we found that in a ChIP assay, the wt hGR exhibited a 3-fold dex-dependent recruitment of GRIP-1 to the MMTV promoter, whereas the triple phosphorylation mutant failed to show any statistically significant dex-mediated difference in GRIP-1 recruitment above vehicle. Interestingly, dex stimulation resulted in more wt GR recruitment, as compared to the hGR triple phosphorylation mutant. This suggests that in addition to a loss of GRIP-1 binding activity, the hGR mutant could also be deficient in DNA binding activity for the MMTV promoter.

Lastly, we investigated the mechanism described above on an endogenous GRE-containing gene in COS-1 cells. We chose the GILZ gene since we had previously shown by ChIP analysis that this gene recruits GRIP-1 to the GRE region in response to dex in U20S cells via the endogenous GR (data not shown). Furthermore, we established here that the maximal level of transactivation of the endogenous GILZ gene is reduced for the over-expressed 3A mutant, showing that the GILZ promoter is sensitive to the phosphorylation status of the GR. Our ChIP results on the endogenous GILZ gene in COS-1 cells show that phosphorylation of the hGR at one or more S residues (from S211, S226, and S203) is required for the interaction of the GR with GRIP-1, but not for recruitment of the GR to the DNA in intact cells on this promoter, consistent with the ChIP results on the MMTV promoter and the in vitro immunoprecipitation data. The finding that the GR recruitment on the endogenous gene is similar for wt and the 3A mutant, unlike the ChIP result obtained on the MMTV promoter, suggests that the role of GR phosphorylation in DNA binding may be promoter-specific.

Our results show for the first time a role for GR phosphorylation in GRIP-1 recruitment. Results from Kino et al., using the same triple hGR mutant construct, suggest that phosphorylation of S203, S211, and/or S226 inhibits the transcriptional activity of the hGR on a MMTV promoter in HCT116 cells via inhibition of recruitment of the histone acetyltransferase (HAT) co-activator, p300, to the GR bound on a MMTV luciferase promoter (*2*). Interestingly, phosphorylation of the hGR at S404, by glycogen synthase kinase 3β (GSK3β), was recently shown to be required for the recruitment of CBP/p300 (*29*). Our finding that the efficacy for transactivation on the GRE-containing TK-luc promoter appears to be insensitive to the phosphorylation status of the GR at these residues suggests that mechanisms other than those involving GRIP-1 recruitment may be involved in this promoter. It would thus appear that phosphorylation of the GR at specific selected serine residues may be a mechanism for rapid differential gene regulation via increasing or decreasing the extent of co-activator, HAT, and/or mediator complex recruitment in a cell- and promoter-specific manner, thereby increasing or decreasing transcription efficacy.

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SUPPORTING INFORMATION AVAILABLE

Specificity of the phosphoserine antibodies as well as the control experiments showing the optimal concentration of α-amanitin and MG132 needed to inhibit transcription and degradation, respectively, and relative amounts of GR and GRIP-1 immunoprecipitated (as a percentage of input) in the co-immunoprecipitation and ABCD assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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