Deciphering the phosphorylation “code” of the glucocorticoid receptor

in vivo

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Summary

The glucocorticoid receptor (GR) is phosphorylated at multiple serine residues in a hormone-dependent manner. Yet, progress on elucidating the function of GR phosphorylation has been hindered by the lack of a simple assay to detect receptor phosphorylation in vivo. We have produced antibodies that specifically recognize phosphorylation sites within human GR at serines 203 and 211 (S203 and S211). In the absence of hormone, the level of GR phosphorylation at S211 is low compared to phosphorylation at S203. Phosphorylation of both residues increased upon treatment with the GR agonist dexamethasone. Using a battery of agonists and antagonists, we found that the transcriptional activity of GR correlates with the amount of phosphorylation at S211, suggesting S211 phosphorylation is a biomarker for activated GR in vivo. Mechanistically, the kinetics of S203 and S211 phosphorylation in response to hormone differ, with S211 displaying a more robust and sustained phosphorylation relative to S203. Analysis of GR immunoprecipitates with the GR phospho-antibodies indicates that the receptor is phosphorylated heterogeneously on S203 in the absence of hormone, whereas in the presence of hormone, a subpopulation of receptors is phosphorylated on both S203 and S211. Interestingly, biochemical fractionation studies following hormone treatment indicate that the S203-phosphorylated form of the receptor is predominantly cytoplasmic, whereas the GR-S211phospho-form is found in the nucleus. Likewise, by immunofluorescence, the S203-phosphorylated GR is located in the cytoplasm and perinuclear regions of the cell, but not in the nucleoplasm, whereas strong phospho-S211 staining was evident in the nucleoplasm of hormone-treated cells. Our results suggest that differentially phosphorylated receptor species are located in unique subcellular compartments, which likely modulates distinct aspects of receptor function.
Introduction

The glucocorticoid receptor (GR) is a phosphoprotein that regulates a wide range of metabolic and developmental processes by controlling the expression of target genes in a hormone-dependent and cell-specific manner (1,2). GR is structurally similar to other members of the nuclear receptor superfamily, in that separate receptor activities such as DNA and ligand binding are localized to distinct regions of the protein (3,4). GR contains a constitutive activation function, AF-1, in the N-terminus, and a ligand-dependent AF-2 at the C-terminus (5). In absence of ligand, the hsp90-based chaperone complex represses GR regulatory activities (6). Hormone binding relieves this repression and results in a conformational change in the receptor, which, in turn, promotes GR DNA binding, association with additional transcriptional regulatory cofactors and regulation of target genes (7).

Although ligand binding is essential for the activation of GR, the receptor is also subject to post-translational modification through phosphorylation (8). GR is phosphorylated in the absence of hormone, with additional phosphorylation occurring in conjunction with agonist, but not antagonist binding. Orti et al. showed that GR is hyperphosphorylated after it has become activated and acquires the ability to bind to DNA (9). It has been suggested that hormone-dependent phosphorylation of GR may determine target promoter specificity, cofactor interaction, strength and duration of receptor signaling, and receptor stability (10).

Bodwell et al. have identified seven phosphorylation sites in the mouse GR (mGR) over-expressed in Chinese hamster ovary cells by direct sequencing of phosphorylated peptides (11). All seven phosphorylated residues are clustered in the N-terminal region of the receptor. Through peptide mapping and mutagenesis studies, our lab has identified four predominant sites of phosphorylation on rat GR (rGR) expressed in mammalian cells and in yeast, that coincide with a subset of sites identified in mGR (12,13). Among them, two sites S224 and S232 corresponding to S203 and S211 in human GR were phosphorylated to a greater extent in the presence of hormone. We have also identified the cyclin-dependent kinases (Cdks) as potential...
kinases that modify S224 and S232 in vitro. Mutations in the Cdk catalytic subunit, p34CDC28 or regulatory cyclin subunits reduced receptor-dependent transcriptional activation in a reconstituted GR signaling system in yeast, indicating that Cdk function is necessary for full receptor-mediated transcriptional enhancement (13).

Previous studies by Mason and Housley suggest that single or multiple phosphorylation site mutations in mouse GR (mGR) had little effect on receptor transcriptional activation, subcellular localization or activity in response to cAMP treatment (14). Similar results were obtained by Almof et al. when phosphorylation site mutants in human GR where analyzed in yeast (15). Webster et al. reported that single or multiple phosphorylation site mutations had little effect on mGR expression, nuclear translocation and transcriptional activation from a complex MMTV promoter (16). Importantly, they also showed that the phosphorylation status of mGR had a substantial effect on transcriptional activation from a GR-responsive reporter containing a minimal E1b-promoter, suggesting that the effect of GR phosphorylation on transcriptional activation appears promoter-specific. However, the mechanism by which phosphorylation affects GR transcriptional regulation remains enigmatic.

To gain further insight into the function of GR phosphorylation in vivo, we have developed antibodies that specifically recognize human GR phosphorylated on either S203 or S211. Using these novel reagents, we examined the kinetics of hormone-dependent GR phosphorylation and the extent of receptor phosphorylation in response to a battery of agonists and antagonists as well as the subcellular localization of the phosphorylated GR in cultured cells and in human tissues. Our findings indicate that GR phosphorylation is a dynamic process, with differentially phosphorylated receptor species partitioned into distinct subcellular compartments which likely affect distinct aspects of receptor function in vivo.
Experimental Procedures

Antibody production

Phosphopeptides were synthesized by Anaspec Inc., (San Jose, CA) that correspond to the following sequences in human GR: 194LQDLEFSSGS\textsuperscript{PO4}PGKE\textsubscript{207} and 202GSPGKETNES\textsuperscript{PO4}PWRS\textsubscript{215}. A cysteine residue was added to the N-terminus of each peptide to facilitate chemical cross-linking. Each phosphopeptide 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 peptides by ELISA. High titer antibodies were further tested on human GR or rat GR expressed in U2OS or SAOS2 cells 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 and were cultured in Dulbecco’s modified Eagle medium (DMEM; Cellgro), supplemented with either 5% or 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah), receptively, 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin (Cellgro). Generation of stable U2OS cell lines ectopically expressing human GR (wild type and phosphorylation site mutants, S203A and S211A) was performed as previously described (17). Cells were seeded at a density between 7 to 9 x 10\textsuperscript{5} cells per 10 cm dish, 18 h prior to transfection. Cells were transfected with using the calcium phosphate method with 10 to 15 µg of wild type pCMV\textsuperscript{Neo-HA-hGR or phosphorylation site mutants pCMV\textsuperscript{Neo-HA-hGRS203A}, pCMV\textsuperscript{Neo-HA-hGRS211A}}. Stable transformants were selected by culturing transfected cells in the presence of 800 µg/ml Geneticin (G418; Invitrogen) for 4-6 weeks. Individual neomycin-resistant 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 dexamethasone (Dex) or equal volume of the ethanol vehicle 1 h prior to lysis. Cells were placed on ice, washed twice in phosphate-buffer saline (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 PMSF, 20 mM β-glycerophosphate, 8 mM sodium pyrophosphate, 1 µg/ml leupeptin, pepstatin A and aprotinin (Roche). 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 2XSDS sample buffer and stored at -20°C. Total lysates from human tissues were prepared from whole-tissue homogenates as described by the manufacturer (Protein Medleys; Clontech).

**Immunoblotting**

Cell extracts or immunoprecipitates containing GR were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and were transferred to Immobilon paper (Millipore Corp., Bedford, MA) at 110V for 80 min in Tris-Glycine transfer buffer. The membranes were blocked overnight in 5% bovine serum albumin (BSA) in Tris-buffered saline pH 7.4 (TBS) (blocking solution) at 4°C, then were incubated in the blocking buffer with primary antibody at room temperature (RT) for 2-4 h using 1:1,000 and 1:10,000 dilution of serum for phospho-S211 (Ab353) and phospho-S203 (Ab211), respectively. Affinity purified phospho-S203 and phospho-S211 antibodies were also tested and results identical to that of the diluted sera were obtained. Endogenous GR was detected using the N499 polyclonal antibody, raised against residues 1-499 of the human GR. 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 (HRP) (Kirkgaard and Perry Laboratories). For blots using the anti-HA-tag monoclonal antibody (α-HA; Roche), an HRP-conjugated goat-anti-mouse IgG secondary antibody was used. Blots were then washed three times for 10 min in TBS-0.1% Triton X-100, twice in TBS and developed using enhanced chemiluminescence (ECL) according to manufacturer’s instructions (Amersham Pharmacia Biotech Inc.). Quantitative analysis of immunoblots was performed using the NIH image software package (version 1.62).

**Immunoprecipitation**

For immunoprecipitation, the GR phosphorylation site-specific antibodies phospho-S203 and phospho-S211 and α-HA were prebound to protein A/G Plus agarose beads (Santa Cruz Biotechnology) in PBS at 4°C for 1.5 hour and washed with PBS twice to remove the unbound antibody. Antibody-coated beads were incubated with 1 mg total protein of U2OS-hGR cell extract at 4°C on a rotator for 3-5 h or overnight. Beads were washed 5 times in PBS and twice in 50 mM Tris pH 7.5 before boiling in 2XSDS sample buffer and stored at -20°C.

**Immunofluorescence**

U2OS-hGR cells were cultured in phenol red-free DMEM containing 10% charcoal-stripped FBS on coverslips coated with poly-D-Lysine and were treated with or without 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 RT, washed 5 times in PBS-0.1% Triton X-100, followed by incubation with goat anti-mouse or goat anti-rabbit fluorescein-conjugated secondary antibody (Vector Labs, Burlingame, CA) diluted in PBS, 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. Cover slips were mounted onto Citifluor (Ted Pella, Redding, CA), and the fluorescein signal was visualized and photographed using a Zeiss Axioplan 2 microscope.


**Immunohistochemistry**

An indirect immunoperoxidase method was used to identify phospho-S211 in normal human tissues arrays prepared by Dr. Herman Yee of NYU School of Medicine, Kaplan Comprehensive Cancer Center Molecular Diagnostics Shared Resource. Tissues were fixed for 2 h in PBS-4% paraformaldehyde at RT, dehydrated through ethanol, cleared in chloroform, and embedded in paraffin. Five-µm tissue sections were serially cut on a microtome and mounted on slides. Sections were dewaxed in xylene, rehydrated, and washed in TBS, pH 7.4. For antigen retrieval, paraffin sections were heated in a microwave oven for 15 min (900 watt, high power) in Target Retrieval Solution (DAKO, Carpinteria, CA), cooled and treated with 3% H2O2 for 15 min, rinsed with H2O and blocked with 20% normal goat serum for 30 min. Sections were incubated with phospho-S211 antibody (1:1,000 dilution) in 10% normal goat serum, washed, and a rabbit secondary biotinylated antibody was added, avidin-biotin complex formed, and developed using diaminobenzidine substrate. Slides were counterstained with hemotaxylin.

**Preparation of cytosolic and nuclear extracts**

Nuclear extracts were prepared as described previously (18). U2OS-hGR cells (a single subconfluent 10 cm dish) were washed twice with cold PBS scraped into a 15 ml conical tube and pelleted by centrifugation at 1,200 rpm for 5 min at 4°C. An equal pellet volume (typically 100 µl) of buffer A (10 mM Hepes pH 7.6, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 0.2 mM sodium metabisulfite, 0.2 mM PMSF and a protease inhibitor cocktail was added, and the cells were allowed to swell on ice for 15 min. Cells were lysed by pushing them rapidly through a 1 ml hypodermic syringe with 25G 3/8” needle at least 5 times. The degree of cell lysis was monitored by light microscopy. The cell homogenate was centrifuged for 20 sec in a microcentrifuge at 12,000 rpm at 4°C. The supernatant was collected and saved as the cytosolic fraction. The nuclear pellet was resuspended in two thirds of cell pellet volume of buffer C (20 mM Hepes pH
7.6, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.2 mM sodium metabisulfate and protease inhibitors) and incubated on ice with stirring for 30 min. The nuclear debris was pelleted by centrifugation for 5 min at 14,000 rpm and the supernatant collected as the nuclear fraction. Each sample was adjusted to equal protein concentration with buffer A or C, and an equal volume of 2XSDS sample buffer was added, boiled for 3 min and stored at -20°C.
Results

Characterization of hGR phosphorylation site specific antibodies

The polyclonal antibodies described in this study, GR phospho-S203 and phospho-S211, were raised against the phosphopeptides LQDLEFSSGS\textsuperscript{PO4}SGKE and GSPGKETNES\textsuperscript{PO4}PWRS corresponding to residues 194-203 and 202-215 of the human GR (Figure 1A). Although GR is phosphorylated at multiple sites, we chose to generate antibodies at phospho-S203 and phospho-S211 since they reside within the minimal activation function 1 (AF-1) region as defined by a GR mutant, termed GR 30IIB, that reduces GR transactivation by disrupting a putative activation surface (19).

The antibodies were tested for their ability to detect GR by immunoblotting of extracts from U2OS cells ectopically expressing an HA-tagged version of human GR (U2OS-hGR) either untreated or treated for 1 h with dexamethasone (Dex). The phospho-S203 antibody recognized GR from both untreated and hormone-treated cells and showed somewhat greater immunoreactivity toward GR from cells stimulated with Dex (Figure 1B, top panel, lanes 1 and 2). The phospho-S211 antibody showed substantial immunoreactivity toward GR from Dex-treated, rather than from untreated cells (Figure 1B, center panel, lanes 1 and 2), even though equal amounts of GR were present as determined by immunoblotting for total GR using HA-epitope residing on the receptor (Figure 1B, bottom panel, lanes 1 and 2). No immunoreactivity toward GR is observed with preimmune sera (not shown). These results indicate that S203 is phosphorylated in the absence and somewhat stronger in the presence of hormone, whereas S211 is phosphorylated predominantly in the presence hormone.

As a control for specificity, we tested the ability of the antibodies to detect phosphorylation-deficient forms of GR S203A and S211A in U2OS cells (U2OS-hGR\textsubscript{S203A} and U2OS-hGR\textsubscript{S211A}). The phospho-S203 antibody did not recognize GR S203A in either the absence or presence of Dex (Figure 1B, top panel, lanes 3 and 4), but still recognized the phosphorylation-deficient GR derivative S211A (Figure 1B, top panel, lanes 5 and 6), to similar extent as wild
type GR. The same relationship holds for the phospho-S211 antibody: it did not detect the hGR S211A mutant (Figure 1B, center panel, lanes 5 and 6), but was reactive with the S203A derivative in a hormone-dependent manner (Figure 1B, center panel, lanes 3 and 4), albeit to a slightly lesser extent than with wild type GR (Figure 1B, center panel, compare lanes 2 and 4). The finding that the S203A mutation reduces phosphorylation at the S211 site suggests an interdependency of these two sites on each other, such that S203 may need to be phosphorylated first, before efficient phosphorylation of S211 can occur. Total amount of GR in each lane was equivalent as revealed by immunoblotting with the HA antibody (Figure 1B, bottom panel). Thus, each antibody recognized its cognate GR phosphorylation site specifically.

**Endogenous hGR phosphorylation in human cells and tissues**

We next examined GR phosphorylation in the human lung carcinoma cells line A549 that expresses endogenous GR. As observed in U2OS cells ectopically expressing hGR, the level of endogenous hGR phosphorylation in A549 cells at S211 is low compared to phosphorylation at S203 in the absence of hormone. Phosphorylation of both residues increased upon treatment with the agonist dexamethasone (Figure 2A). Thus, the phospho-S203 and phospho-S211 antibodies are capable of detecting phosphorylation of endogenous GR from a cultured cell line.

We also investigated hGR phosphorylation from lysates of human tissues by immunoblotting (Figure 2B). Of the four GR responsive tissues examined, thyroid, prostate, lung and liver, abundant GR phospho-S203 and phospho-S211 reactivity was observed in the prostate and liver, whereas weak phospho-GR immunoreactivity was observed in the thyroid. The same pattern holds true for total GR. We were unable to detect phospho-GR in the lung sample, even upon prolonged exposure (not shown), although a small amount of total GR was observed. Thus, prostate, thyroid and liver from normal human tissues contain phospho-GR isoforms.

To determine which cell types display the S211-phosphorylated form of GR, serial sections of normal human tissues were examined by immunohistochemistry with either the GR
phospho-S211 antiserum or an antibody that recognizes GR independent of its phosphorylation state (total GR) (Figure 2C). A section of a human lymph node shows strong phospho-S211 immunoreactivity within the nuclei of lymphocytes, a pattern reflected in the staining for total GR. Similarly, the nuclei of thyroid cells react with the phospho-S211 antibody, whereas both the cytoplasm and nuclei of islet cells of the pancreas stain positive. In contrast, lung tissue shows little phospho-S211 immunoreactivity within the alveolar cells, despite immunoreactivity of their cytoplasm the total GR antibody. This low level of immunoreactivity is consistent with the results from the immunoblotting analysis of lung extracts (Figure 2B). Although alveolar cells showed no detectable phospho-S211 immunoreactivity, macrophages resident in the lung appear to contain phospho-S211 GR, indicating that the staining procedure worked. Thus, the S211-phosphorylated, hormone-activated form of GR is readily detected in vivo in a subset of normal human tissues.

Effects of agonists and antagonists on hGR phosphorylation of S203 and S211

The influence of different glucocorticoid agonists and antagonists on phosphorylation of GR was also examined. Three kinds of glucocorticoid agonists, Dex, Prednisolone and Fluocinolone, and two types of glucocorticoid antagonists, RU486 and ZK299 were tested for their ability to induce GR phosphorylation and affect GR-mediated transcriptional activation after 1 hour of treatment. As shown in Figure 3, Dex, Prednisolone and Fluocinolone promoted S211 phosphorylation, whereas RU486 only minimally stimulated phosphate addition to S211, a pattern mirrored in the GR transcriptional activation assay. For the S203, Dex and RU486 induced roughly the same amount receptor phosphorylation (Figure 3A). ZK299 had almost no effect on the phosphorylation of either S203 or S211. In addition a 10-fold molar excess of RU486 over Dex could effectively block the Dex-dependent stimulation of S211 phosphorylation. A 10-fold excess of ZK299 was not as effective at blocking Dex-dependent phosphorylation of GR, presumably owing to its lower affinity for the receptor. These results suggest that the GR-
mediated transcriptional activity correlates with GR phosphorylation, suggesting an association between receptor-dependent transcriptional enhancement and S211 phosphorylation.

**Kinetics of S203 and S211 phosphorylation in response to hormone**

To examine the kinetics of phosphorylation of S203 and S211, we performed immunoblots of U2OS-hGR extracts made at different times of Dex treatment using the phospho-S203 and phospho-S211 antibodies (Figure 4). The HA antibody was used to determine the total amount of GR. The relative amount of immunoreactivity for each time point was quantitated. Hormone treatment had little effect on total GR at 1 h, but resulted in a time-dependent decrease in GR that started at 2 h of hormone treatment and continued for 12 h; such decrease in GR protein level has been previously attributed to increased GR mRNA and protein turnover (20,21). Total GR was reduced by nearly 80% by 12 h of continuous hormone treatment. As shown in Figure 4A, phosphorylation at S211 increased rapidly within the first hour of Dex treatment, reaching a plateau within 20 to 40 min of hormone treatment. On a longer time scale, S211 phosphorylation remained high for 6 h, after which the apparent signal decreased progressively from 7 to 12 h. Phosphorylation of S203 increased at a rate similar to that of S211 within the first hour of hormone treatment (Figure 4B, top panel). When normalized to GR protein at each time point, S203 phosphorylation increased at 1 h of Dex treatment and remained almost unchanged relative to total GR between 1 and 12 h (Figure 4C). This result indicates that S203 phosphorylation reaches a maximum at 1 h of Dex treatment and then parallels the kinetics of down regulation of GR. In contrast, S211 phosphorylation increased more markedly and at a slower rate than S203 phosphorylation, reaching a maximum at 6 h of hormone treatment and decreased at the same rate as total GR after 10 h (Figure 4C). Thus, hormone-dependent phosphorylation at S211 is more robust and sustained than S203 phosphorylation.
Heterogeneity of GR phosphorylation

Results from the immunoblot analysis indicate that GR is phosphorylated at S203 and S211 in the presence of Dex. However, this finding does not distinguish between receptors with a single phosphate moiety at each site or receptor molecules that are phosphorylated at S203 and S211 simultaneously. To address this issue, GR was immunoprecipitated with phospho-S203, phospho-S211 or HA from lysates of U2OS-hGR cells that had been cultured with or without Dex for 1 h. The immunoprecipitates were analyzed by immunoblotting with HA and the GR phospho-specific antisera. As expected, the HA antibody immunoprecipitated an equivalent amount of GR from Dex-treated or untreated cells (Figure 5; bottom panel, lanes 1 and 2). The phospho-S211 antiserum preferentially immunoprecipitated GR from Dex-treated cells (Figure 5, lanes 3 and 4), whereas the phospho-S203 antibody immunoprecipitated GR from hormone-treated and untreated cells, with more GR being precipitated from the Dex-treated sample (Figure 5, lanes 5 and 6). The immunoprecipitation results are consistent with the immunoblotting of whole cell extracts, and support the idea that phosphorylation at S211 is largely hormone-dependent, whereas phosphorylation of S203 occurs in the absence of hormone but is increased upon hormone stimulation. Importantly, the hGR S211 immunoprecipitate was recognized by the phospho-S203 antibody and visa versa, indicating that GR can be phosphorylated on S203 and S211 simultaneously (Figure 5, top and center panels, lanes 3-6). Therefore, a population of GR molecules that is doubly phosphorylated on both S203 and S211 resides in vivo.

The fact that phosphorylation at S203 is increased in response to hormone suggest that not all GR is basally phosphorylated on S203. Indeed, if GR were homogeneously phosphorylated at S203, then we would anticipate that total GR and phospho-S203 immunoprecipitates would produce the same immunoreactivity when blotted with GR phospho-S203 antibody. In contrast, if GR were heterogeneously phosphorylated at S203, then we would expect to see less GR phospho-S203 immunoreactivity from immunoprecipitates of total GR versus phospho-S203, which is observed experimentally (Figure 5, compare lanes 1 and 5; top
Therefore, within a population of GR molecules in the absence of hormone \textit{in vivo}, some receptors are phosphorylated at S203, whereas others remain unphosphorylated.

\textbf{The subcellular location of hGR phospho-S203 and phospho-S211 isoforms}

To determine the subcellular location of GR phosphorylation, we prepared cytosolic and nuclear fractions from U2OS-hGR cells either untreated or treated with Dex for 1 h. In the absence of hormone, GR was detected exclusively in the cytosol fraction, while after a 1 h Dex treatment the receptor was distributed equally between cytosol and the nuclear fractions (Figure 6A). Interestingly, phosphorylation of S203 in the absence and presence of Dex was detected exclusively in the cytosolic fraction of U2OS cells, whereas phosphorylation of S211 was observed in both the cytosolic and nuclear fractions (Figure 6A, lanes 1-4). To ensure the fidelity of the fractionation procedure, we examined each fraction for the cytosolic and nuclear marker proteins, RhoGDI\(\alpha\), a Rho guanine nucleotide dissociation inhibitor, and TATA-box binding protein (TBP), a member of the TFIIID transcriptional regulatory complex. As expected, RhoGDI\(\alpha\) was found predominantly in the cytosolic fraction, whereas TBP immunoreactivity was observed primarily in the nuclear fraction. Thus, the procedure accurately separated the cytoplasmic and nuclear fractions. These results suggest that S203 and S211 are phosphorylated in the cytoplasm and that the S211-phosphorylated form of the receptor resides in the nucleus after a 1 h hormone treatment.

Immunofluorescence was performed with phospho-S203 and phospho-S211 antibodies and yielded results consistent with the fractionation studies (Figure 6B). In untreated U2OS-hGR cells, the S203-phosphorylated form of the receptor was observed primarily in the cytoplasm and perinuclear region of the cell, but not in the nucleoplasm. Upon hormone treatment, the same pattern holds true, with stronger perinuclear phospho-S203 immunoreactivity observed. Virtually no phospho-S211 immunoreactivity was observed in the cytoplasm or nucleus of untreated cells. In contrast, strong phospho-S211 staining was evident in the nucleoplasm of hormone-treated
cells after a 1 h hormone treatment, with diffuse phospho-S211 immunoreactivity observed in the cytoplasm. This staining is specific to phospho-GR since no immunofluorescence was observed above background in U2OS-hGR$_{S203A}$ and U2OS-hGR$_{S211A}$ cells when examined with the phospho-S203 and phospho-S211 antisera, respectively (not shown). Together, these results suggest that the S203-phosphorylated form of GR is predominantly cytoplasmic and perinuclear, whereas the S211-phosphophorylated species is strikingly nuclear after hormone stimulation.

We also examined the subcellular distribution of the phospho-S203 and phospho-S211 hGR isoforms in A549 cells (Figure 7). As was observed in U2OS-hGR cells, strong phospho-S211 immunoreactivity was observed in the nuclei of hormone treated cells, whereas the S203-phosphorylated form of the receptor was distributed primarily throughout the perinuclear region. These results corroborate our findings in U2OS-hGR cells and suggest that different phosphorylated isoforms of GR localize to distinct subcellular compartments.
Discussion

We have generated antibodies that specifically recognize hGR phosphorylated on S203 and S211. Both antibodies react with specific GR phosphorylation states by immunoblotting or immunoprecipitation, suggesting that the antibodies detect the phosphorylated receptor in either its native or denatured form (Figures 1 and 5). There appears to be a significant basal level of GR phosphorylation at S203 but not S211, and phosphorylation of both residues was increased upon treatment with Dex. This is consistent with results of $^{32}$P metabolic labeling studies (13,22). The enhancement of phosphorylation at S203 is about 2-fold, whereas for S211 it is approximately 10-fold after a 1 h of Dex treatment (Figure 4).

We also found a strong correlation between S211 phosphorylation of GR and receptor transactivation; potent agonists, such as Dex and Prednisolone, induced robust GR phosphorylation at S211, while mixed agonists/antagonists, such as RU486, elicited only modest receptor phosphorylation (Figure 3). Effect of RU486 on GR phosphorylation at S211, suggests that the kinase phosphorylating this residue may be recruited to the receptor via the ligand binding domain, with RU486 affecting this association. Recently, Chen, et al. demonstrated that AF-1 activity of the estrogen receptor alpha (ER) is modulated by phosphorylation as a result of the ligand-dependent recruitment of a Cdk through the receptor’s ligand-binding domain (23). Whether GR phosphorylation of S211 operates through a similar mechanism remains to be tested.

Conceivably, phosphorylation of S211 may alter the receptor conformation or modulate interactions with cofactors that facilitate transactivation. Consistent with this latter notion, GR interaction with the AF-1 cofactor DRIP150 is reduced when S211 is mutated to alanine (A. Hittelman and M. Garabedian, unpublished observation). In addition, the phospho-S211 antibody was capable of recognizing GR within normal human tissues by immunohistochemistry. Thus, phosphorylation of S211 may represent an important biomarker for the hormone-activated form of GR in vivo.
The time course of S203 and S211 phosphorylation in response to continuous Dex treatment showed that S211 phosphorylation was more sustained than S203 phosphorylation, suggesting that phosphorylation at S203 is more labile than phosphorylation at S211. Whether phospho-S203 and phospho-S211 are differentially sensitive to the same phosphatase, represent targets for distinct phosphatase(s), or whether partitioning of S211 into the nucleus protects it from dephosphorylation or promotes hyperphosphorylation remains to be determined.

It is interesting to note that down-regulation of GR occurs at the same rate as S203 phosphorylation, suggesting that modification of this residue might be a determinant of ligand-dependent down-regulation of GR. This would be consistent with the finding of Webster et al. that characterized phosphorylation site mutations that stabilize the GR and decreased its ligand-dependent down-regulation (16).

Subcellular localization of the phospho-GR isoforms assessed biochemically indicate that upon hormone stimulation the S203 phosphorylated forms of the receptor is detected in the cytoplasm, whereas GR-P-211 is evident in both the cytoplasmic and nuclear fractions (Figure 6A). Interestingly, by immunofluorescence, the S203 phosphorylated form of the receptor is confined primarily to the cytoplasm and perinuclear regions of the cell, whereas the receptor phosphorylated at S211 localizes to the nucleoplasm and cytoplasm after a 1 h hormone stimulation (Figure 6B). These findings suggest that unique phosphorylated forms of the receptor are distributed to distinct subcellular compartments: the GR phospho-S211 form corresponds to the nuclear transcriptionally active subpopulation of the receptor, whereas the GR phospho-S203 form is perinuclear, possibly affiliated with receptor function(s) at the cytoplasmic/nuclear border, such as nuclear import or export. Alternatively, GR phosphorylation may represent a quality control step or checkpoint that insures that the receptor is properly configured for events such as cofactor binding. Thus, specific subpopulations of GR with different phosphorylation patterns may modulate distinct aspects of receptor function in vivo.
The first phosphorylation state-specific antibodies against a steroid receptor, the progesterone receptor (PR), were described in an elegant set of experiments by Clemm et al. (24). As with GR, the PR hormone-dependent phosphorylation sites are regulated differentially over time. However, unlike GR, PR phosphorylated forms examined appear to reside in the nucleus. Whether this reflects differences in the phosphorylation sites examined between GR and PR, or receptor kinases and phosphatases that target these receptors will require further study.

Based on our results, we propose a model of GR modulation by phosphorylation whereby in the absence of hormone, the phosphorylated subpopulation of GR is mostly modified on S203; hormone treatment promotes phosphorylation of both S203 and S211, causing an increase in doubly phosphorylated form GR-P-S203/S211 (Figure 8). We speculate that this doubly phosphorylated version of GR “primes” the receptor for S203-P dephosphorylation, resulting in the mono-S211 phosphorylated version of the receptor that accumulates in the nucleus. This mechanism is analogous to substrate “priming” by kinases, such as glycogen synthase kinase-3, where a prior phosphorylation event generates the substrate for subsequent modification (25). As time progresses, GR-P-S211 phosphorylation and GR-P-S203 dephosphorylation continues increasing the ratio of GR-P-S211 to GR-P-S203. The S211-phosphorylated form enters the nucleus where phosphorylation remains high, however, exit from the nucleus leads to S211 dephosphorylation, resulting in a “naked” receptor subspecies that lacks a phosphate adduct on either S203 or S211. The receptor can then adopt two fates: it can either be recycled or degraded. “Naked” receptors may reassemble into chaperone-bound aporeceptor complexes, which would facilitate S203 phosphorylation, thus re-establishing the GR-P-S203 form. Receptors that do not associate with the hsp90 chaperone complex are subject to degradation. This notion is consistent with the previous studies, whereby treatment of cells with the hsp90 inhibitor geldanamycin promoted GR degradation by the proteosome (26). Although further experiments will be necessary to distinguish whether phosphorylation promotes receptor recycling or degradation, this
cycle of phosphorylation and dephosphorylation likely plays a key role in modulating the hormonal response \textit{in vivo}.

Protein phosphatases types 1, 2A and 5 (PP1, PP2A, and PP5) have been shown to associate with GR and affect receptor function and phosphorylation (27-29). Inhibition of phosphatase activity by okadaic acid results in GR hyperphosphorylation, a redistribution of the receptor from the nucleus to the cytoplasm, and the inability of GR to re-enter the nucleus (30). We speculate that inhibition of the phosphatase activity leads to an accumulation of the doubly phosphorylated form GR-P-S203/S211 in the cytosol by virtue of decreased entry into or increased exit from the nucleus.

Our findings also shed light on the kinases that target the GR. Biochemical fractionation studies suggest that GR phosphorylation at both S203 and S211 occurs in the cytoplasm, with the S211-phosphorylated form accumulating the nucleus. Previous results from our laboratory have shown that cyclin E/Cdk2 and cyclin A/Cdk2 phosphorylate GR at S203 and that cyclin A/Cdk2 targets S211 \textit{in vitro} (13). Since the active forms of these kinases are predominantly nuclear (31), it is unlikely that these represent the receptor kinases that promote phosphorylation at S203 and S211 in the cytoplasm. However, it is conceivable that two distinct kinases, one cytoplasmic and the other nuclear, target S211 and that cyclin A/Cdk2 is the receptor kinase that promotes S211 phosphorylation in the nucleus. In light of the dual phosphorylation of ER$\alpha$ S118 by Erk and Cdk (23,32), it is possible that GR may be similarly targeted by multiple kinases depending upon the subcellular compartment the receptor occupies.

We suggest that differential modification of GR by phosphorylation induces distinct conformations and/or influences the receptor association with additional coregulatory proteins that modulate GR transactivation and stability. Phosphorylation of GR represents a unique modification “code” that is deciphered in the nucleus and cytoplasm to differentially affect receptor function. Subsets of phosphorylated receptor species with distinct functions may emerge as a novel and general mechanism governing steroid hormone receptor action.
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Figure Legends

Figure 1. Structure of hGR and specificity of phospho-S203 and phospho-S211 antibodies.

A) Functional domains and phosphorylated residues of the hGR. Shown is a schematic representation of hGR with phosphorylation sites corresponding to those in mGR as determined by Bodwell et al. (20). The sequences of the hGR phosphopeptides used to produce the antibodies. B) Immunoblotting of hGR with phospho-S203 and phospho-S211 antibodies are shown. Whole cell extracts prepared from U2OS cells expressing an HA-tagged hGR (U2OS-hGR), either wild type or phosphorylation site mutant derivatives S203A or S211A, untreated or treated with 100 nM Dex for 1 h were analyzed by immunoblotting with phospho-S203 (top panel), phospho-S211 (center panel) or HA antibodies (bottom panel) as a measure of total GR.

Figure 2. Expression of endogenous phospho-GR in human cultured cells and tissues.

A) Whole cell extracts prepared from A549 cells expressing endogenous hGR were untreated (-) or treated with 100 nM Dex for 1 h and analyzed by immunoblotting with phospho-S203 (top), phospho-S211 (center) or the phosphorylation state-independent antibody, GR N499 (bottom) as a measure of total GR. B) Extracts from human tissues (50 µg/lane, Protein Medleys, Clontech) were analyzed by immunoblotting with phospho-S203 (top), phospho-S211 (center) or the phosphorylation state-independent antibody, GR N499 (bottom). C) Expression of endogenous GR phospho-S211 in human tissues. The gallery of images (400X) shows immunohistochemical analysis of paraffin-embedded human tissues treated with the GR phospho-S211 antibody or a GR antibody that recognizes GR in a phosphorylation-state independent manner (total GR). GR phospho-S211 reactivity, seen as brown staining, is observed within the nuclei of human lymphocytes, thyroid cells, islet cells of the pancreas, but not in the alveolar cells of the lung (left panel), despite the presence of GR in all tissues examined (right panel). No staining above background is observed when preimmune serum is used (not shown).
Figure 3. Effects of agonists and antagonists on GR phosphorylation and transcriptional activation.

A) U2OS-hGR were treated with ethanol (-) or the ligands indicated (100 nM) for 1 hour and whole cell extracts were prepared. For antagonism experiments, a 10-fold excess of RU486 (1 µM) or ZK299 (1 µM) were added simultaneously with 100 nM Dex for 1 h prior to cell lysis. Equal amounts of protein from each treatment were analyzed by immunoblotting with either phospho-S203, phospho-S211 or HA antibodies, reflective of the amount of total GR present in each lane. B) U2OS-hGR 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 determined.

Figure 4. Kinetics of S203 and S211 phosphorylation in response to Dex treatment.

U2OS-hGR cells were treated with ethanol (-) or Dex (100 nM) for the time indicated. Whole cell lysates were prepared, normalized and analyzed by immunoblotting with phospho-S203, phospho-S211 or HA antibodies. Immunoblot images of GR phosphorylated at S203 and S211 (A) in the first hour of Dex treatment or (B) throughout 12 h of continuous Dex treatment; the total GR in each sample was determined by immunoblotting for HA. C) Quantitative analysis of immunoblot results in panel B normalized to total GR detected by HA. The data shown are from a single experiment that is representative of at least three independent experiments.

Figure 5. Phosphorylation of GR on both S203 and S211 in response to Dex treatment and heterogeneous phosphorylation of S203 in untreated cells.

Immunoprecipitation of the phosphorylated forms of GR by phospho-S203 and phospho-S211 antibodies. U2OS-hGR cells were treated with either ethanol or 100 nM Dex for 1 h and whole cell extracts containing equal amounts of protein were immunoprecipitated with using either
phospho-S203, phospho-S211 or HA antibodies. Immunoprecipitates were analyzed by immunoblotting with phospho-S203, phospho-S211 and HA antibodies.

**Figure 6.** The subcellular localization of phospho-S203 and phospho-S211 GR in U2OS-hGR cells.

A) Cytosolic (C) and nuclear (N) extracts were prepared from U2OS-hGR cells treated with ethanol or 100 nM Dex for 1 h. Equal amounts of protein from each fraction were analyzed by immunoblotting with either phospho-S203, phospho-S211 or HA antibodies. Fractions were immunoblotted for cytosolic and nuclear marker proteins, RhoGDIα and TBP, respectively, to ensure the integrity of the fractionation procedure. B) U2OS-hGR cells treated exactly as above, with either ethanol (0) or 100 nM Dex for the times indicated and were fixed, and the subcellular location of GR was examined by indirect immunofluorescence using phospho-S203, phospho-S211 or HA antibodies as a measure of total GR. The data shown are from a single experiment that is representative of at least three independent experiments.

**Figure 7.** The subcellular distribution of endogenous phospho-S203 and phospho-S211 GR in A459 cells.

A549 cells, which contain endogenous GR, were treated with either ethanol (-) or 100 nM Dex (+) for 1 h, fixed, and the subcellular location of GR was examined by indirect immunofluorescence using the phospho-S203 and phospho-S211 antibodies or the phosphorylation state-independent antibody, GR N499 (total GR).

**Figure 8.** A model for GR-dependent phosphorylation

In the absence of hormone, the phosphorylated subpopulation of GR is mostly modified on S203 (orange), a population of unphosphorylated receptor molecules may also be present (blue); hormone treatment promotes phosphorylation of both S203 and S211 (red), causing an increase in
doubly phosphorylated form GR-P-S203-S211 (green, orange and red). As time progresses, GR-
P-S203 undergoes dephosphorylation such that the ratio of GR phosphorylated on S211 to those
phosphorylated on S203 increases. Differential modification of GR by phosphorylation induces a
distinct conformation and/or influences the receptor association with additional coregulatory
proteins that modulate GR transactivation, stability and subcellular location.

**Supplemental Data Figure 1**

U2OS-hGR, U2OS-hGR_{S203A} and U2OS-hGR_{S211A} cells were treated with 100 nM Dex for 2 h,
fixed, and the subcellular location of GR was examined by indirect immunofluorescence using
phospho-S203, phospho-S211 or HA antibodies as a measure of total GR. The micrograph of the
hGR_{S211A} subcellular location (bottom panel, lower right hand side) using the phospho-S211
antibody is exposed four times longer than the comparable image from the U2OS-hGR cells to
show outline of the cells in the field.
Figure 1

A

hGR

S113 S141 S224 S317 S508

AF-1 DBD LBD (AF-2)

S203 S211

L-Q-D-L-E-F-S-S-G-S-P-G-K-E

* 194 207 202 215

G-S-P-G-K-E-T-N-E-S-P-W-R-S

S203 S211

B

Immunoblot

U2OS-hGR:

Dex: WT 203A 211A

GR-P-203

GR-P-211

HA

(Total GR)

1 2 3 4 5 6
Fig. 2

A

| A549 | Dex: | -- | + |
|------|------|----|----|
| GR-P-203 |
| GR-P-211 |
| Total GR |

B

| Thyroid | Prostate | Lung | Liver |
|---------|----------|------|-------|

C

GR-P-211

lymph node

thyroid

pancreas

lung

lymphocyte

islet

alveolar

thyroid
Fig. 3

A

Ligands: Ethanol, Dex, RU486, Prednisolone, Fluocinolone, ZK299, RU486+Dex, ZK299+Dex

GR-P-203

GR-P-211

HA (Total GR)

B

GR Transactivation

RLU x1000

Ligands: Ethanol, Dex, RU486, Prednisolone, Fluocinolone, ZK299, RU486+Dex, ZK299+Dex
Fig. 4

A  Dex:  0  5  10  20  40  60 min

GR-P-203

GR-P-211

HA  (Total GR)

B  Dex:  0  1  2  4  6  7  8  10  12 h

GR-P-203

GR-P-211

HA  (Total GR)

C  Normalized GR phosphorylation

![Graph with Time Dex (hour) on the x-axis and Relative Mean on the y-axis, showing data points for GR-P-211 and GR-P-203]
Fig. 5

**Immunoprecipitation**

| IP:  | HA | GR-P-211 | GR-P-203 |
|------|----|----------|----------|
| Dex: | -- | +        | --       |

**Blot**

- GR-P-203
- GR-P-211
- HA (Total GR)
Fig. 6

A

| Fractions: | C | N | C | N |
|-----------|---|---|---|---|
| GR-P-211  |   |   |   |   |
| GR-P-203  |   |   |   |   |
| HA (Total GR) |   |   |   |   |
| TBP       |   |   |   |   |
| RhoGDI    |   |   |   |   |

Dex: -- +

B

| Dex:    | 0 | 0.5h | 1h | 2h | 4h | 6h |
|---------|---|------|----|----|----|----|
| total GR|   |      |    |    |    |    |
| GR-P-211|   |      |    |    |    |    |
| GR-P-203|   |      |    |    |    |    |
Fig. 7

total GR

GR-P-211

GR-P-203

Dex: - +

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Fig. 8
Supplemental Data Figure 1

|          | U2O  | S-hGR |
|----------|------|-------|
| Total GR | WT   | S203A | S211A |
| GR-P-203 |      |       |       |
| GR-P-211 |      |       |       |
