Mouse Glucocorticoid Receptor Phosphorylation Status Influences Multiple Functions of the Receptor Protein*

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Although studies have shown that the mouse glucocorticoid receptor (mGR) contains eight phosphorylation sites (Bodwell, J. E., Ortí, E., Coull, J. M., Pappin, D. J. C., Smith, L. I., and Swift, F. (1991) J. Biol. Chem. 266, 7549–7555), the effect of phosphorylation on receptor function is unclear. We have examined the consequences of single or multiple phosphorylation site mutations on several properties of mGR including receptor expression, ligand-dependent nuclear translocation, hormone-mediated transactivation, ligand-dependent down-regulation of mGR, and receptor protein half-life. Mutations had little effect on receptor expression, subcellular distribution, ligand-dependent nuclear translocation, or on the ability to activate hormone-mediated transcription from a complex (murine mammary tumor virus) promoter. In contrast, the phosphorylation status of the mGR had a profound effect on the ability to transactivate a minimal promoter containing simple glucocorticoid response elements after hormone administration. Similarly, ligand-dependent down-regulation by glucocorticoids of both receptor mRNA and protein was abrogated in mutants containing three or more phosphorylation site alterations. Finally, we show that the phosphorylation status of mGR has a profound effect on the stability of the glucocorticoid receptor protein. Receptors containing seven or eight mutated sites have a markedly extended half-life and do not show the ligand-dependent destabilization seen with wild type receptor. These data show that receptor phosphorylation may play a crucial role in regulating receptor levels and hence control receptor functions.

The glucocorticoid receptor (GR) is a member of a family of intracellular ligand-inducible transcription factors termed the steroid/vitamin D/retinoic acid superfamily (2, 3). All of these receptors share certain structural and functional features, such as an amino-terminal transactivation domain, a central Zn$^{2+}$-finger DNA binding domain, and a carboxyl-terminal ligand binding region. Upon exposure to a specific ligand, the receptor undergoes a transformation process and binds with high affinity to its cognate sequence-specific DNA response element. After DNA binding, the receptor interacts with the basal transcription complex and alters transcription of hormone sensitive genes (4). Many of these proteins, including GRs, are phosphorylated and can become hyperphosphorylated after binding by ligand (1, 5, 6). The role of receptor phosphorylation in receptor function, however, is controversial. Earlier studies suggested that mutation of single or multiple phosphorylated sites in mouse or human GR had little effect on the ability of these mutants to activate transcription (7, 8). However, promoter complexity and context may affect the ability of various phosphorylated forms of the GR to regulate transcription. Therefore, it remains possible that other transcription factors harbored in complex promoters could compensate for potential impaired effects of dephosphorylated GRs.

Another feature shared among some members of the steroid receptor family is that of hormone-mediated down-regulation (9). Our laboratory has shown previously that GR down-regulation occurs primarily at the level of transcription, is restricted to ligands of GRs, and is reversible on hormone withdrawal (10, 11). The genetic elements responsible for hormone-mediated autoregulation reside within the exons of the GR cDNA (12). In addition, GR protein exhibits ligand-dependent destabilization, but little is known about GR turnover or what stimuli alter receptor half-life. However, recent evidence suggests that phosphorylation plays a role in the turnover of other proteins (13–15).

Here we report that decreased phosphorylation in the mouse GR (mGR) decreases transactivation of a hormone-responsive simple promoter. In addition, we show that hormone-dependent autoregulation of the mGR mRNA and protein is abolished in mutants bearing three or more substitutions. Strikingly, receptor half-life is greatly increased with decreased phosphorylation, suggesting that phosphorylation is involved in receptor turnover.

**EXPERIMENTAL PROCEDURES**

*Materials—Unless otherwise specified, all reagents were purchased from Sigma. Dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxyprogesterone) was purchased from Steraloids (Wilton, NH). The anti-peptide polyclonal antibody to GR (generated by guests) was purchased from Schleicher & Schuell. 20-cm TLC Silica Gel 60 sheets were purchased from ICN Radiochemicals (Irvine, CA). [3H]Chloramphenicol (40–60 Ci/mmol) was purchased from DuPont NEN. Biotinylated and horseradish peroxidase-conjugated antibodies were purchased from ICN. Protran nitrocellulose BA85 was purchased from Whatman. 20 × 20-cm TLC Silica Gel 60 sheets were purchased from EM Separation Technology (Gibbstown, NJ).

Expression Vectors and Reporter Plasmids—Wild type and mutant mGRs were all expressed in the same pSV2SRE vector whose expression is driven by an SV40 promoter. Generation of mutant mGRs in which alanine was substituted for serine or threonine has been described previously (17) (see Table I). The MMTV-CAT reporter plasmid.
**Glucocorticoid Receptor Phosphorylation**

| Mutant glucocorticoid receptors | Substituted amino acids |
|---------------------------------|-------------------------|
| 212A                            | Serine 212 to alanine    |
| 220A                            | Serine 220 to alanine    |
| 234A                            | Serine 234 to alanine    |
| 212/234A                        | Serine 212, 234 to alanine |
| 220/234A                        | Serine 220, 234 to alanine |
| A5                              | Serine 212, 220, 234 to alanine |
| A4                              | Serine 150, 212, 220, 234 to alanine |
| A5 + 412A                       | Serine 150, 212, 220, 234, 412, and threonine 159 to alanine |
| A7                              | Serine 150, 212, 220, 234, 315, 412, and threonine 159 to alanine |
| A8                              | Serine 122, 150, 212, 220, 234, 315, 412, and threonine 159 to alanine |

(pGMCS), kindly provided by Dr. Don DeFranco, and the minimal reporter plasmid GRE2-TATA-CAT, have been described previously (18, 19).

**Immunohistochemistry**—The method for assessing the subcellular distribution of both wild type mGR and phosphorylation mutants transiently transfigured into COS-1 cells has been described previously (20). Briefly, COS-1 cells (African green monkey kidney, ATTC) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 9 mg/ml glucose, 100 Ui/ml penicillin, 100 μg/ml streptomycin, and supplemented with 2% glutamine and 10% of a 1:1 mixture of fetal calf/calf serum (Irvine Scientific, Santa Ana, CA). Cultures were passed every 3–4 days and were maintained in culture for no longer than 15 passages. Cells were transfected by the DEAE-dextran method of Sompayrac and Danna (21) as modified by Gorman (22). Cells were incubated with the appropriate DNA/DEAE dextran mixture for 3 h and placed in Dulbecco’s modified Eagle’s medium supplemented with steroid-stripped fetal calf serum and incubated further at 37 °C for 24 h. Transfected cells were placed in two-chamber glass slides, incubated for an additional 24 h, and then treated with 100 nm dexamethasone or vehicle for 1–2 h. Cells were fixed and processed for immunohistochemical staining as described previously (23).

**Chloramphenicol Acetyltransferase (CAT) Assays**—Transfections and CAT assays were performed according to the calcium phosphate method described by Gorman (22). COS-1 cells were cotransfected with either wild type or various phosphorylation mutants and either the MMTV-CAT reporter pGMCS or the GRE2-TATA-CAT reporter plasmids. After transfection, cells were either treated with hormone (100 nm dexamethasone) or vehicle and incubated for 24 h. Cell extracts were then analyzed for CAT activity. For our assays, the concentration of radiolabeled chloramphenicol required were determined in prior studies to ensure that the percent conversion of radiolabeled chloramphenicol to the acetylated forms was in the linear range. After hormone treatment, cells were treated with 100 nM dexamethasone or vehicle for 1–2 h. Cells were fixed and processed for immunohistochemical staining as described previously (23).

**RESULTS**

**Wild Type and Phosphorylation Mutants Are Expressed in Transiently Transfected COS-1 Cells**—Although the wild type and all of the phosphorylation mutants were transfigured from the same backbone vector, pSV2twRec (17), we examined the expression obtained in transiently transfected COS-1 cells to determine if phosphorylation sites influenced receptor expression. Western blot analysis of total cell receptor protein using an epitope-purified polyclonal anti-GR antibody revealed that all of the mutant receptors were expressed in the cells (Fig. 1). It should be noted that no receptor could be detected when backbone vector alone was transfected into the COS-1 cells (not shown). Furthermore, no novel degradation products were noted for any of the phosphorylation mutants. The faster migrating 90-kDa band seen for wild type and all mutants probably represents a ubiquitous GR degradation product. The most substituted forms of the mGRs, A7 and A8, migrated only slightly faster than the wild type receptor. This observation is in agreement with results obtained by Almof et al. (8), who were able to visualize only a modest size difference in a human GR mutant when five phosphorylated sites had been abolished in an expression system producing a truncated GR.

**Phosphorylation Mutants Translocate to the Nucleus upon Exposure to Hormone**—We next analyzed subcellular localization of receptor in the absence and presence of hormone. Immunohistochemistry of COS-1 cells transiently transfected with either wild type or various phosphorylation mutants of the mGR further demonstrated that all of the receptors were expressed in the COS-1 cell line. By immunohistochemistry, relatively similar levels of expression for the wild type and all of the phosphorylation mutants were observed. The subcellular distribution of the mGR and phosphorylation mutants in the transient transfection system revealed that in the absence of hormone, all receptors have a cytoplasmic location (Fig. 2, **CON panels**). However, we see greater variability in the initial subcellular distribution of the mouse GR compared with human GR (28). Evaluation of the effect of hormone on nuclear trans-
location of wild type and phosphorylation mutants revealed that all of the mGRs translocate to the nucleus (Fig. 2, DEX panels). Thus, hormone-mediated nuclear translocation occurred regardless of the phosphorylation state of the receptor.

**Transcriptional Activation by mGR Phosphorylation Mutants**—The ability of the various phosphorylation mutants to activate transcription of hormone-inducible reporter genes was next assessed. Previously, Mason and Housley (7) reported that phosphorylation mutants activated transcription of a glucocorticoid-inducible MMTV-CAT reporter plasmid as effectively as wild type mouse GR. Similarly, Almo¨f et al. (8), using glucocorticoid-responsive elements (GREs) linked to the complex thymidine kinase promoter, observed comparable activation between wild type and phosphorylation mutations of the human GR. We also examined the ability of mouse phosphorylation mutants to activate the complex promoter from the glucocorticoid responsive MMTV-CAT plasmid pGMCS. The wild type mGR demonstrated a 12-fold induction over control when 100 nM dexamethasone was added to the culture medium for 24 h (Fig. 3A). All of the mutants showed an 8–20-fold induction of the reporter gene; but, in agreement with previous work (7, 8), none of these inductions was significantly different from the wild type receptor.

We next considered if promoter context was an important component in the efficacy of various phosphorylated forms of mGR to activate transcription. To answer this question, we used the reporter plasmid GRE2-TATA-CAT (19) which contains two copies of the GRE from the tyrosine aminotransferase gene positioned just upstream of the minimal adenovirus E1b TATA sequence (Fig. 3B). Strikingly, all but one of the phosphorylation mutants we tested exhibited a decrease in its ability to transactivate transcription after hormone administration (Fig. 3B). These transactivations were only 25–50% of that seen for wild type mGR. Interestingly, the decreases observed were not additive as more phosphorylated sites were mutated, and clearly, all phosphorylation mutants activated transcription. Similar results were obtained when we used a lower dose (1 nM) of hormone to activate the transfected receptors (not shown). Therefore, the differences observed were with the relative po-

**Loss of Autoregulation in Phosphorylation Mutants of the mGR**—Since we determined that promoter context is an important feature in the ability of phosphorylation-deficient mutants to activate transcription, we wished to determine if the phosphorylation state of mGR could affect a gene that is negatively regulated in
response to glucocorticoids. Accordingly, we examined the effect of phosphorylation of mGR on the down-regulation of its own gene. Previously we determined that the GR is down-regulated in response to cognate ligands and that this down-regulation occurs at the level of transcription (11). The genetic elements responsible for the down-regulation are contained within the GR coding region (cDNA) (12). Consequently, one can measure the effect of phosphorylation on GR gene expression by transiently transfecting the expression vectors of different phosphorylation-deficient mGRs into COS-1 cells and determining the relative levels of mGR mRNA and protein following administration of hormone. When wild type mGR was transfected into COS-1 cells the mGR mRNA typically showed a decrease to less than 50% of control levels after treatment with 100 nM dexamethasone replicating previous results seen with human GR (12) (Fig. 4A). For those receptors with a single (212A, 220A, 234A) or with two (212/234A or 220/234A) mutated phosphorylation sites the effect of hormone on receptor mRNA levels was marginally (55–67% of control levels) different from wild type mGR (45–50% of control levels) after 100 nM dexamethasone treatment (Fig. 4A). Nevertheless, in all these cases, the differences in single and double substitution mutant GR mRNA levels after hormone treatment were not as great as seen for wild type mGR mRNA. Receptor constructs A3, A4, A5, A5+412A, A7, and A8 (Table I) all showed a substantial reduction in hormone-mediated down-regulation of the mouse GR gene. These data argue that the phosphorylation state of the mGR receptor is extremely important in attenuating rates of receptor transcription.

**Loss of Autoregulation in Phosphorylation Mutants of the mGR Protein—**Receptor protein levels were next examined to determine if they were affected by mutation of mGR phosphorylated sites. In agreement with the mGR mRNA data (Fig. 4A), the wild type protein levels decreased to 45% of control levels after treatment with dexamethasone. Neither single or double mutations of phosphorylated sites had significant effects on down-regulation of the mGR protein (Fig. 4B). However, multiple (A3–A8) mutations caused a dramatic loss of hormone-mediated receptor down-regulation, with hormone-treated protein levels 90–160% of untreated receptor levels (Fig. 4B). These results are consistent with those for mGR mRNA where the multiple mutants were not affected by hormone treatment (Fig. 4B). Interestingly, the dexamethasone-treated A5, A7, and A8 mutants showed levels of mGR protein even higher than controls (resistant to proteolytic degradation) than the phosphorylated form.

**Phosphorylation Status Affects Receptor Half-life—**To evaluate the effect that phosphorylation may have on receptor protein half-life, wild type, A7, and A8 phosphorylation mutants were transiently transfected into COS-1 cells. The cells were then treated with 1 μM cycloheximide for 1 h and then left untreated (control), or hormone was added. Relative receptor levels were measured by Western analysis at various times after cycloheximide treatment. Fig. 5A shows that control wild
Quantitated using a densitometer and normalized to b-actin mRNA. Data shown are representative of four experiments. Levels of mGR mRNA were quantitated using a densitometer and normalized to b-actin mRNA. Control (CON, black bar) levels were expressed as 100 with dexamethasone (DEX, open bars) treatment represented as a mean percent of control ± S.E. Panel B, whole cell lysates were prepared, and the proteins were separated on a 7.5% acrylamide gel and transferred to a nitrocellulose membrane. The mGR was detected after incubation with an anti-GR antibody following the standard protocol for an ECL Western (Amersham). Western data were quantitated using the video image analysis software, NIH 1.56 for the Power PC. The data represent at least three replicates from separate transfection studies. Controls (black bar) were represented as 100 with dexamethasone (stippled bar) treatment presented as a mean percent of the control ± S.E.

**Discussion**

The role that phosphorylation contributes to the GR function has been the subject of several recent studies and is controversial (1, 7, 8). Initially, we examined if the phosphorylation status of the GR had any effect on hormone-induced nuclear translocation. None of the mutants we studied showed a diminution of nuclear translocation. In fact, the most mutated form of the mGR (A8) we tested in which all eight phosphorylation sites were abolished, showed translocation properties similar to those of the wild type receptor. It should be noted that the mGR shows more variability in the initial subcellular distribution than its human counterpart (20, 28). However, once the receptors were exposed to hormone, the translocation to the nucleus was complete.

All of the phosphorylation mutants we studied showed a hormone-inducible transcriptional response from the MMTV-CAT reporter system we tested. To date, the other reported studies have used either an MMTV-CAT reporter (7) or a vector with two GREs linked to a complex thymidine kinase promoter (8) and have shown that phosphorylation mutants were capable of eliciting a transcriptional response from a hormone-inducible reporter gene. However, we wished to analyze the transactivation potential of the phosphorylation mutants using a simpler system where additional transcription factors could not compensate for a loss of necessary phosphorylation sites. Therefore, we also tested a hormone-inducible reporter plasmid containing a minimal promoter GRE2-TATA-CAT (19). With the exception of the single substitution 234A the transactivation potential of the phosphorylation mutants was decreased to 25–35% of wild type. Our results suggest that promoter complexity has a significant bearing on transactivation by dephosphorylated receptors. These data imply that when the receptor interacts with the basal transcription complex in the absence of other ancillary factors, the phosphorylation state of the receptor is important in determining the magnitude of the hormone-induced response. A recent report by Kato et al. (29) showing that the human estrogen receptor must be phosphorylated at Ser-118 to achieve full response of the estrogen receptor activation function suggests that a similar status may exist for mGR. Similarly, Weigel and colleagues (30) have shown that phosphorylation mutants of the progesterone receptor give an attenuated response with a hormone-responsive reporter. Weigel and colleagues (30) have shown that phosphorylation mutants of the progesterone receptor provide an attenuated response to hormone treatment. However, our studies show that hormone-mediated autoregulation of the GR gene is abolished in mutants missing three or more phosphorylation sites. Thus, in this model, repression of gene
expression by GR may be dependent on phosphorylation. It is unknown at this time if the effects on GR down-regulation occur by direct interaction of GR with the RNA polymerase II complex (31, 32) or with specific steroid receptor coactivators (33) or directly with the GR gene (11, 12). We speculate that phosphorylation may direct the receptor to act as an activator or repressor of gene transcription. Phosphorylation may represent an adaptive mechanism whereby GR can differentially regulate its own gene expression based on the availability of active kinases and phosphatases in different cell types and tissues.

The observation that the dephosphorylated forms of the GR, A7 and A8, showed higher protein levels when treated with hormone in the absence of increased mRNA levels suggests that this receptor ligand complex is more stable than the phosphorylated wild type mGR. Therefore, we determined receptor half-lives of wild type and both A7 and A8 mutants in the presence and absence of dexamethasone. The wild type mGR when transfected into COS-1 cells showed a half-life similar to what Dong et al. (36) observed for rat HTC cells. In a study using dense amino acids (37) in rat GH cells, GRs in untreated cells had a half-life of 19 h, whereas GR in cells treated with trimacincalone acetonide had a half-life of 9.5 h. In our studies, the wild type control cells showed a half-life of approximately 18 h whereas the dexamethasone-treated (100 nM) cells had a half-life of 8–9 h (Fig. 5A). These results clearly imply that there is a hormone-mediated destabilization of the GR. With the A7 and A8 mutants, without hormone, the half-lives were increased to 25 h (Fig. 5B) and 29 h (Fig. 5C), respectively, and hormone treatment had only a slight or no effect. These results are especially interesting in light of the fact that other laboratories have found that phosphorylation targets certain proteins for proteolysis. For example, Lanker et al. (34) showed recently that rapid degradation of the G1 cyclin Cln2 occurred after phosphorylation by a cyclin-dependent protein kinase, and Chen et al. (35) demonstrated that the nuclear factor KB suppressor IxBo needs to be phosphorylated for kinase-dependent ubiquitination and subsequent protein degradation to occur. Therefore, phosphorylation of the GR is likely to be necessary for receptor turnover. It should be noted that it has been shown that upon hormone binding the GR becomes hyperphosphorylated (6), supporting the notion that phosphorylation could target the receptor for hormone-mediated degradation. Furthermore, these results immediately imply that the phosphorylation mutants may have a poorer transactivation potential than observed, as a potentially greater concentration of receptors would be present in hormone-treated cells transfected with phosphorylation mutants. Thus, the small effects seen on transactivation of complex promoters as well as the larger effects seen on simple promoters are likely to be under-estimates. These results demonstrate for the first time that the phosphorylation status of the mGR receptor has profound effects on different receptor functions.

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