Phosphorylation and Inhibition of Rat Glucocorticoid Receptor Transcriptional Activation by Glycogen Synthase Kinase-3 (GSK-3)

SPECIES-SPECIFIC DIFFERENCES BETWEEN HUMAN AND RAT GLUCOCORTICOID RECEPTOR SIGNALING AS REVEALED THROUGH GSK-3 PHOSPHORYLATION

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Transcriptional activation by the glucocorticoid receptor (GR) is regulated by both glucocorticoid binding and phosphorylation. The rat GR N-terminal transcriptional regulatory domain contains four major phosphorylation sites: threonine 171 (Thr171), serine 224 (Ser224), serine 232 (Ser232), and serine 246 (Ser246). We have previously demonstrated that Ser224 and Ser232 are phosphorylated by cyclin-dependent kinases, while Ser232 is phosphorylated by the c-Jun N-terminal kinase. We report here that the remaining GR phosphorylation site, Thr171, is a target for glycogen synthase kinase-3 (GSK-3) in vitro and in cultured mammalian cells. Increasing GSK-3 activity through its overexpression in cultured cells inhibits GR transcriptional enhancement, an effect dependent upon Thr171. Correspondingly, overexpression of a constitutively active form of the GSK-3 inhibitor, protein kinase B/Akt, increases GR transcriptional enhancement. Overexpression of GSK-3 had no effect on GR-mediated transcriptional repression of AP1-dependent gene expression. Importantly, transcriptional activation by the human GR (hGR), which contains an alanine (Ala149) at the position equivalent to Thr171 in rat GR, is not affected by GSK-3 overexpression. Introduction of a threonine residue at this position (A150T) establishes GSK-3-mediated inhibition of hGR transcriptional activation. These findings demonstrate species-specific differences in GR signaling, as revealed through GSK-3 phosphorylation, which suggests that GR function in rodents may not fully recapitulate receptor action in humans and that hGR is capable of adopting the GSK-3 signaling pathway through a somatic mutation.

Glucocorticoid hormones control cellular proliferation and metabolism through their association with the glucocorticoid receptor (GR), a member of the intracellular receptor superfamily of transcriptional regulatory proteins (1). Upon glucocorticoid binding, GR enters the nucleus, associates with specific DNA sequences termed glucocorticoid response elements (GREs), and increases transcriptional initiation from nearby promoters. GR can also repress transcription mediated by the heterodimeric AP1 transcription factor complex (c-Jun and c-Fos) (2). Although glucocorticoids act as the primary signal in activating GR’s transcriptional regulatory functions, GR-mediated transcriptional activation is also modulated by phosphorylation (3–5).

Rat GR isolated from cultured mammalian cells or ectopically expressed in yeast (Saccharomyces cerevisiae) is phosphorylated on four major residues (6). These sites cluster to the N-terminal transcriptional regulatory domain and include threonine 171 (Thr171), serine 224 (Ser224), serine 232 (Ser232), and serine 246 (Ser246) (Fig. 1A). Each of these residues is followed by a proline, thereby forming a motif phosphorylated by a family of serine/threonine-proline-directed kinases that includes the cyclin-dependent kinases (Cdk), the mitogen-activated protein kinases, and glycogen synthase kinase-3 (GSK-3). Differential phosphorylation at these sites both positively and negatively regulate GR transcriptional activation. Positive regulation is accomplished by cyclin-Cdk complexes: cyclin E-Cdk2 phosphorylates Ser224, while cyclin A-Cdk2 phosphorylates both Ser224 and Ser232. Mutations at these sites, or of particular Cdk genes in yeast, reduce GR-dependent transcriptional activation, suggesting that phosphorylation of Ser224 and Ser232 is required for full GR transcriptional enhancement (7). In contrast, phosphorylation of Ser246 by c-Jun N-terminal kinase, a member of the mitogen-activated protein kinase family, inhibits GR transcriptional activation (8).

The remaining GR phosphorylation site, Thr171, also resides in a motif recognized by serine/threonine-proline-directed kinases. However, our previous studies indicate that neither the Cdns, nor c-Jun N-terminal kinase efficiently phosphorylate Thr171 in vitro. Furthermore, phosphorylation of Thr171 is evident in both serum-deprived quiescent and serum-stimulated proliferating cells (8), suggesting that Cdns and c-Jun N-terminal kinase are unlikely to phosphorylate Thr171 in vivo, since these kinases are largely inactive in serum-starved, nonproliferating cells. GSK-3, on the other hand, is active throughout the cell cycle, as well as in serum-deprived cells (9). Thus, GSK-3 may represent the GR kinase that phosphorylates Thr171.

GSK-3 was originally isolated as the kinase that phosphorylates glycogen synthase, the rate-limiting enzyme of glycogen synthesis. PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; wt, wild type.
Regulate factors involved in cellular proliferation including metabolism. GSK-3 has been implicated in cell fate determination have implicated GSK-3 in pathways other than glycogen metabolism present in yeast (S. cerevisiae and Schizosaccharomyces pombe) (12, 13), Dictyostelium discoideum (14), Drosophila melanogaster (15, 16), and Xenopus laevis (17–19).

Recent studies in Dictyostelium, Xenopus, and Drosophila have implicated GSK-3 in pathways other than glycogen metabolism. GSK-3 has been implicated in cell fate determination have implicated GSK-3 in pathways other than glycogen metabolism present in yeast (S. cerevisiae and Schizosaccharomyces pombe) (12, 13), Dictyostelium discoideum (14), Drosophila melanogaster (15, 16), and Xenopus laevis (17–19).

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Here we examine whether rat GR is a substrate for GSK-3 and investigate the consequences of GSK-3 activation and inhibition on GR transcriptional regulation in cultured mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Purification of Receptor Derivatives and in vitro Kinase Assays**—A wild type rat GR derivative containing amino acids 106–318 or receptor mutant with a single amino acid substitution T171A, and a wild type human ER derivative containing estrogen receptor (ER) X-terminal amino acids 1–121, were expressed in S. cerevisiae as glutathione S-transferase (GST)-fusion proteins (GST-GR106–318 and GST-ER1–121) exactly as described previously (7). The most concentrated fractions (1 mg/ml) were used as substrates for the in vitro kinase assays.

GSK-3 activity was, in turn, activated through an association with lipid products generated by phosphorylatedinsulin-kinase B/Akt, an enzyme that phosphorylates and inhibits GSK-3 (24).

**Plasmids**—pcMV-wt GR and pcMV-GR T171A expression plasmids were used to produce rat GR, and 

**Cell Lines and Treatments**—U-2 OS human osteosarcoma cells (ATCC HTB 96) and human HeLa cervical carcinoma cells (ATCC CCL 2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone), 50 units/ml each of penicillin and streptomycin, and 2 mM l-glutamine (Life Technologies, Inc.). Rat pheochromocytoma PC-12 cells (ATCC CRL-1721) were maintained in MEM supplemented with 10% horse serum, 2 mM L-glutamine, 50 units/ml each of penicillin and streptomycin, and 2 mM l-glutamine. All hormone treatments were done in DMEM, 10% FBS containing either 100 nM glucocorticoid dexamethasone (resuspended in 100% ethanol) or an identical volume of 100% ethanol.

**Transient Transfections and Reporter Activity Assays**—U-2 OS cells were plated on 60-mm dishes in DMEM, 10% FBS. One hour prior to transfection cells were refed with fresh medium and transfected with the indicated plasmids via the calcium phosphate precipitation method as described elsewhere (35). Eight hours later, cells were washed three times with prewarmed phosphate-buffered saline to remove calcium phosphate precipitates, allowed to recover overnight in DMEM, 10% FBS and incubated with fresh medium containing 100 nM dexamethasone where indicated, for an additional 8 h.

HeLa and PC-12 cells were plated in 60-mm dishes, washed once with serum-free medium and transfected with the indicated plasmids using 20 μl of LipofectAMINE reagent (Life Technologies, Inc.) in a total volume of 2.5 ml of serum-free phenol red-free DMEM per 60-mm dish according to the manufacturer’s instructions. Three hours post-transfection 2.5 ml of DMEM, 10% FBS was added to each dish and cells were harvested the next day. The next day cells were refed with fresh DMEM, 10% FBS with 100 nM dexamethasone or identical volume of 100% ethanol and incubated for an additional 8 h.

Transfected cells were washed twice in phosphate-buffered saline and harvested in IX Reporter lysis buffer (Promega). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1

**Supernatant**—The supernatant containing the digested peptides was evaporated to dryness. Peptides were resuspended in 0.5 ml of water, dried, washed again, and dissolved in 10 μl of electrophoresis buffer I, pH 1.9 (15% acetic acid, 5% formic acid). Peptides were electrophoresed in the same buffer on a thin layer chromatography plate (microcrystalline cellulose acetate Coomassie blue as indicator; Kodak, Denville, NJ) for 8 V with 3 V in the 35 min. Plates were then dried, subjected to ascending chromatography in the second dimension for 3.5 h with 37.5% butanol, 25% pyridine, and 7.5% acetic acid, air-dried, and exposed to film (34).

For phosphoammonic acid analysis, 1-P-labeled receptor was transferred to Immobilon paper (Millipore Corp.), and the GR band was excised from the membrane, and hydrolyzed in 100 μl of 6 N HCl (Pierce) by heating to 110 °C for 60 min. Samples were washed twice in 0.5 ml of water, dried, and resuspended in 8 μl of electrophoresis buffer I. The hydrolysates were spotted onto a TLC plate, along with phosphoammonic acid standards (1 μl of mixture of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma), 1 mg/ml each), and resolved in the first dimension by electrophoresis at 1500 V for 20 min in electrophoresis buffer I, and in the second dimension by electrophoresis at 1300 V for 16 min in buffer II, pH 3.4 (5% acetic acid, 0.5% pyridine). After drying, plates were sprayed with 0.25% w/v ninhydrin in acetone and developed at 70 °C for 10 min to visualize the phosphoammonic acid standards, and autoradiography was performed.

**Site-directed Mutagenesis**—Site-directed mutagenesis of the human GR alanine 150 to threonine was performed using Stratagene’s Quick Change site-directed mutagenesis procedure and high fidelity Pfu DNA polymerase, according to the manufacturer’s instructions, with the following oligos: 5′-GCTGTGTCTGTACCCCCACAGAGAAG-3′ and 5′-CTTTCTGTGTGGGTTACAGACGGC-3′.

**Plasmids**—pcMV-wt GR and pcMV-GR T171A expression plasmids were used to produce rat GR, and Xc3-GR reporter plasmid, containing two consensus GREs upstream of thymidine kinase promoter (−109) linked to a luciferase gene was used to assay GR transcriptional activity. An XAPITL reporter plasmid, containing a single AP1 binding site upstream of the thymidine kinase promoter fused to a luciferase gene, was used to assay transcriptional repression. pcDNA3-hGR and pcDNA3-hGR A150T plasmids expressed the human wild type and the alanine to threonine mutant GRs, respectively. pcMV5-GR-GSK-3β-expressing HA-tagged GSK-3 and pcMV6-GR-Akt plasmid expressed a constitutively active myristylated HA-tagged form of Akt (28). A pcMV5 empty vector was used to equalize the total amount of DNA transfected in each experiment. pcMV-LacZ plasmid produced β-galactosidase.

**In Vitro Kinase Assays**—All kinetic assays were performed using 20 μl of an enzyme reaction mix containing 100 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 1 mM dithiothreitol, 10 mM ATP, 0.2 mg/ml bovine serum albumin, 10 μg/ml each of aprotinin, pepstatin A, leupeptin, 10 μg/ml pepsin, and 10 μg/ml trypsin. The reactions were performed in 1 ml of kinase buffer containing 25 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 50 units/ml of V8 protease (endoproteinase Glu-C, Bohl, Bohringer Mannheim), and were incubated at 30 °C for 15 min, unless otherwise noted. The reactions were terminated by the addition of 2× SDS sample buffer and run on 10% SDS-PAGE. The gels were stained with Coomassie Blue to visualize the phosphoprotein standards, and autoradiography was performed.
RESULTS

GSK-3 Phosphorylates the Rat Glucocorticoid Receptor in Vitro at Threonine 171—To examine whether GSK-3 can utilize the rat GR as a substrate in vitro, we tested purified rabbit GSK-3β for its ability to phosphorylate a GST-GR fusion protein containing the receptor residues 106 through 318 (GST-GR<sub>106–318</sub>). We compared GR phosphorylation by GSK-3 to that of the established GST-GR substrate, the transcription factor c-Jun. Fig. 1B demonstrates that GSK-3 phosphorylates the GST-GR<sub>106–318</sub> and GST-c-Jun<sub>1–223</sub> in vitro with similar efficiency. In contrast, under the same experimental conditions GSK-3 failed to phosphorylate an ER derivative encompassing residues 1 through 121 (GST-ER<sub>1–121</sub>), which contains three serine-proline phosphorylation sites at Ser<sup>104</sup>, Ser<sup>106</sup>, and Ser<sup>118</sup> and has been shown previously to be substrate for cyclin dependent kinase (CDK) and protein phosphatase 1 (PP1). Thus, it appears that the N-terminal transcriptional regulatory domain of rat GR is a substrate for GSK-3 in vitro.

Two-dimensional phosphopeptide mapping was used to identify specific residues that are phosphorylated by GSK-3 in vitro based on the known positions of each GR phosphopeptide when digested with V8 (Glu-C) protease (Fig. 2A). The GST-3-phosphorylated GST-GR<sub>106–318</sub> fusion protein was digested with V8 protease and the resulting peptides were resolved by electrophoresis and chromatography, yielding a single phosphopeptide that corresponds to a GR peptide containing phospho-Ser<sup>171</sup> (Fig. 2B). Since Thr<sup>171</sup> is the only threonine residue phosphorylated in the rat GR, we would expect phosphoamino acid analysis to detect only phosphothreonine if Thr<sup>171</sup> is indeed the target for GSK-3 phosphorylation. As shown in Fig. 2C, phosphothreonine is the only phosphoamino acid present in GR when phosphorylated by GST-3 in vitro. To further confirm that GR Thr<sup>171</sup> is the primary target for GSK-3 phosphorylation
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...in vivo, we have generated a GST-GR<sub>106–318</sub> fusion protein with a single threonine to alanine substitution at Thr<sup>171</sup> (T171A) and subjected it and the wild type (wt) GR to an in vitro kinase assay with GSK-3. Fig. 2D demonstrates that the T171A amino acid substitution abolished GR phosphorylation. Taken together, these findings indicate that Thr<sup>171</sup> is the site of phosphorylation by GSK-3 in the rat GR.

Ectopic Expression of GSK-3 Inhibits GR Transcriptional Activation, but Not Repression, in Cultured Mammalian Cells—Since GSK-3 is expressed ubiquitously and is constitutively active in virtually all mammalian cell lines, we examined whether GSK-3 overexpression alters GR-dependent transcriptional activation. We transiently co-expressed GSK-3, rat GR, and a GR-responsive reporter plasmid containing two consensus GREs upstream of the luciferase gene, in U-2 OS human osteosarcoma cells, which lack endogenous GR. Fig. 3A demonstrates that increasing amounts of transfected GSK-3 inhibit GR hormone-dependent transcriptional enhancement in a dose-dependent manner, with the maximal dose of GSK-3 used reducing GR transcriptional activity by over 50% compared with control cells transfected with the vector alone. This effect likely represents an underestimate of the total impact of GSK-3 on GR activity since the results are obtained in a cell line expressing endogenous GSK-3. The reduction in GR activity was not due to the inhibition of GR protein expression, since the steady-state level of GR was not affected by the GSK-3 overexpression (Fig. 3B). Inhibition of GR activity by ectopically expressed GSK-3 was also observed in rat PC-12 cells (Fig. 3C), which express GR endogenously, suggesting that the effect of GSK-3 on GR transcriptional activity extends to multiple cell types.

Since hormone-activated GR can also repress transcription (2, 38, 39), we examined the effect of GSK-3 on GR-mediated transcriptional repression of AP1-dependent transcription by transiently co-expressing GSK-3, the rat GR, and a reporter gene containing a single AP1 site upstream of the luciferase gene in U-2 OS cells. In the absence of GR activation by the glucocorticoid dexamethasone, AP1 activity was reduced as a result of GSK-3 overexpression (Fig. 3D), consistent with the inhibitory phosphorylation of c-Jun by GSK-3 (20, 23). In contrast, no significant changes in either wt (Fig. 3D) or T171A (not shown) GR-mediated repression of AP1 activity in the presence of dexamethasone was observed upon GSK-3 overexpression, suggesting that phosphorylation of Thr<sup>171</sup> by GSK-3 does not alter GR’s ability to repress transcription.

On the basis of our in vitro findings indicating that Thr<sup>171</sup> is the site of GSK-3 phosphorylation in rat GR, we anticipate that GSK-3-mediated reduction in GR transcriptional activity should be dependent on the presence of Thr<sup>171</sup> target site. To examine this hypothesis, we transiently transfected either the wt GR or GR T171A mutant into U-2 OS cells and assessed the effects of GSK-3 overexpression on GR transcriptional enhancement. As shown in Fig. 4A, the T171A substitution relieved the inhibitory effect of GSK-3 on GR activity. Furthermore, the T171A mutation increased GR transcriptional enhancement over the wt GR by 26 and 40% in the absence and presence of dexamethasone, respectively.
presence of co-transfected GSK-3, respectively, consistent with the inhibitory effect of GSK-3 on rat GR transcriptional activation and reflecting the loss of inhibition by endogenous GSK-3 (Fig. 4A). The steady state level of GR protein was unaffected for both the mutant and wt GR in both the presence and absence of ectopic GSK-3 expression (Fig. 4B). Thus, rat GR is phosphorylated by GSK-3 at Thr171 in vitro, and mutation of this site to alanine eliminates the effect of GSK-3 overexpression on rat GR transcriptional activation in cultured mammalian cells.

Akt, an Inhibitor of GSK-3, Increases GR Transcriptional Enhancement—We next asked whether a decrease in GSK-3 activity would increase GR transcriptional activation. Since no GSK-3-deficient cell line is available, we chose to inhibit GSK-3 activity by using Akt, a protein kinase that phosphorylates GSK-3 and inhibits its catalytic activity. We overexpressed a constitutively active membrane-targeted myristylated form of Akt (28) in U-2 OS cells and measured GR-dependent transcriptional activation. GR transcriptional enhancement in the presence of ectopically expressed Akt was increased to nearly 300% compared with that of control cells receiving an empty expression vector (Fig. 5A). Akt also increased transcriptional activation of endogenous GR in rat PC-12 cells, and the rat GR introduced into GR-negative SAOS2 human osteosarcoma cells (data not shown), suggesting that this effect is not confined to a single cell type. Thus, activation of Akt increases GR transcriptional activation.

Our hypothesis that Akt acts by phosphorylating and inactivating GSK-3, and thus, relieving the inhibitory effect of GSK-3 on GR transcriptional enhancement, predicts that the effect of Akt should also be dependent on Thr171, the target of GSK-3 phosphorylation. Fig. 5A demonstrates that transcriptional enhancement by the rat GR T171A mutant was largely insensitive to Akt overexpression, suggesting that Akt’s effect on GR transcriptional activation is accomplished principally through inhibition of GSK-3-mediated phosphorylation of Thr171. There is, however, a small (~25%) effect of Akt on GR transcriptional activity that is independent of Thr171, which suggests that Akt may also be modifying accessory factors involved in GR transcriptional regulation. The Akt-mediated increase of GR transcriptional activity was not a result of the changes in steady-state GR protein levels (Fig. 5B). Thus, overexpression of Akt increases GR transcriptional activation, in a Thr171-dependent manner, suggesting that Akt’s effect is accomplished primarily through inhibition of GSK-3.

Inhibition of GR Transcriptional Activity by GSK-3 Is Spe-
cies-restricted—The analysis of GRs isolated and sequenced from different species demonstrates that the majority of GR phosphorylation sites are evolutionarily conserved. For example, serine residues 224, 232, and 246 in the rat GR are conserved among human, primates, mouse, guinea pig and Xenopus receptors (40–44). In contrast, Thr171 is conserved between rat, mouse, and guinea pig GR, but corresponds to an alanine (Ala150) residue in the hGR, suggesting that hGR may be insensitive to signaling by GSK-3. To examine this possibility, we tested the effects of GSK-3 overexpression on the transcriptional activation of endogenous hGR in HeLa cells, and in U-2 OS cells, where hGR was introduced ectopically. Transient overexpression of GSK-3 failed to inhibit hGR transcriptional activation in HeLa cells (Fig. 6A). Since our transfection studies with the rat GR were performed in U-2 OS cells, we had to eliminate the possibility that these cells contain a specific co-factor necessary for GSK-3 action on GR. Fig. 6B demonstrates that in U-2 OS cells, no decrease in the hGR transcriptional activity was observed upon GSK-3 overexpression. Thus, under conditions identical to those used for the experiments with the rat GR (Fig. 3A), GSK-3 did not inhibit transcriptional enhancement by hGR, revealing species-specific differences in GR signaling.

We next asked whether replacing the alanine residue at position 150 with a threonine (A150T) in the hGR would confer sensitivity to GSK-3. To test this hypothesis, we have constructed a hGR A150T mutant and assessed the effects of GSK-3 overexpression on the transcriptional activity by this hGR mutant in U-2 OS cells. As shown in Fig. 6C, the transcriptional activity of the hGR A150T mutant decreases as a function of increasing amounts of ectopically expressed GSK-3, whereas no such inhibition was noted with wt hGR. Thus, introduction of a threonine residue into hGR at a position equivalent to Thr171 in the rat GR, results in a GSK-3-dependent inhibition of the hGR transcriptional activation.

**DISCUSSION**

We have demonstrated that GSK-3 phosphorylates the rat GR at Thr171 in vitro. In cultured mammalian cells, overexpression of GSK-3 inhibits GR transcriptional activation, while decreasing GSK-3 activity, through expression of the GSK-3 inhibitor, Akt, increases GR transcriptional enhancement. GR-mediated repression of AP1-dependent transcriptional activity, however, was not affected by GSK-3 overexpression. A threonine to alanine mutation at Thr171, the site of rat GR phosphorylation by GSK-3 in vitro, eliminates the effect of GSK-3 on rat GR transcriptional enhancement. Although the effect of GSK-3 overexpression on GR-mediated transcriptional activation was relatively modest (~50%), this likely represents an underestimate of the impact of GSK-3 on GR, since the studies were performed in cell lines containing active endogenous GSK-3. Our in vitro phosphorylation and mapping studies, coupled with activity assays using GR mutants, strongly suggest that GSK-3 phosphorylates rat GR at Thr171, and as a consequence, reduces GR transcriptional activation.

The mechanism by which GSK-3 phosphorylation of Thr171 decreases GR transcriptional activity is unclear. GSK-3 phosphorylates and inactivates other regulatory factors including c-Myc, c-Jun, NF-ATc, and b-catenin. Although the mechanisms of c-Myc and c-Jun inactivation by GSK-3 phosphorylation is unknown, GSK-3 phosphorylation of b-catenin targets it for degradation (45, 46), while GSK-3 phosphorylation of NF-ATc promotes its export from the nucleus (47). It is doubtful, however, that either of these established mechanisms explain GSK-3 regulation of GR, since 1) neither GSK-3 nor Akt overexpression alter steady state GR protein levels, and 2) increased export of GR from the nucleus would also affect GR-dependent transcriptional repression, which has not been observed in our experiments. Alternatively, GSK-3-mediated phosphorylation of rat GR at Thr171 may disrupt protein-protein interactions that favor GR transcriptional enhancement, or recruit inhibitory proteins that antagonize GR-dependent transcriptional activation, hypotheses that are currently being tested.

Given the high degree of conservation between GRs from...
different species, it is particularly striking that the hGR does not contain a site of GS3-mediated phosphorylation, thereby making hGR insensitive to GS3 overexpression. However, when an alanine residue at the position homologous to Thr171 in rat GR is replaced with a threonine, transcriptional activation by the hGR A150T mutant becomes sensitive to GS3 overexpression. Sequence comparison between GRs isolated from different species shows that the primary amino acid sequence surrounding and including rat GR Thr171 (residues 164 through 173) is conserved among rodents, including rat, mouse, and guinea pig (40, 41, 43). The equivalent region from human, squirrel monkey, owl monkey, and cotton-top tamarin GR remains conserved among primates, but has diverged from rodents (42, 48, 49). Why this region of GR has diverged between primates and rodents, while the other major phosphorylation sites (Ser224, Ser232, and Ser246) are conserved remains unclear, but likely reflects alternative strategies adopted by each species to regulate GR action.

The differences in GR primary amino acid structure and signaling between rodents and humans may contribute to the greater sensitivity of murine lymphocytes to glucocorticoid-induced apoptosis relative to human cells. It is conceivable that GS3-mediated inhibition of GR transcriptional activation in rodents results in the reduced expression of a putative survival factor induced by GR. Recently, the cyclin-dependent kinase inhibitor p21cip1 has been shown to be a GR-responsive gene (49–51) and forced expression of p21 can block apoptosis (52). Thus, p21 expression protects cells from apoptosis, and as such, can be considered a survival factor. It is tempting to speculate that inhibition of GR by GS3, and the subsequent lack of p21 induction, may facilitate apoptosis in murine but not human lymphocytes. It would be interesting to replace mouse GR with that of the human GR in vivo and examine whether the glucocorticoid-induced apoptosis of murine lymphocytes expressing hGR still occurs. We speculate further that a threonine at position 150 in hGR would result in greater glucocorticoid sensitivity compared with an alanine at this position.

Our findings demonstrate species-specific differences in human and rat GR signaling, which suggest that studies on GR function in mice and rats may not fully translate into hGR activity. In addition, our results indicate that hGR is capable of adopting the GS3 signaling pathway through a somatic mutation, which antagonizes hGR-dependent transcriptional activation. It would be informative to examine whether alanine to threonine substitutions at residue 150 in hGR are present in glucocorticoid-sensitive, but absent in glucocorticoid-resistant, malignancies.

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