**Glucocorticoid Signaling Is Perturbed by the Atypical Orphan Receptor and Corepressor SHP**

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SHP (NROB2) is an atypical orphan nuclear receptor that lacks a DNA-binding domain but contains a putative ligand-binding domain. Previous studies have revealed that SHP interacts with a variety of nuclear receptors and inhibits their transcriptional activity, thereby acting as a corepressor. In this report we identify the glucocorticoid receptor (GR) as a novel downstream target receptor for SHP inhibition. SHP potently inhibits dexamethasone-induced transcriptional GR activity in mammalian cells, and the inhibition involves a functional second NR-box within SHP. Interestingly, this motif shows a high homology with the NR-box in the glucocorticoid and eAMP-inducible GR coactivator PGC-1, indicating similar binding specificity and shared target receptors. We show that SHP antagonizes PGC-1 coactivation and, in addition, we identify the PGC-1-regulated phospho(enol)pyruvate carboxykinase (PEPCK) promoter as a novel target promoter for SHP inhibition. This implies a physiologically relevant role for SHP in modulating hepatic glucocorticoid action. Furthermore, when coexpressing green fluorescent protein-tagged GR together with SHP, an intranuclear redistribution of GR was observed. As inhibition-deficient SHP mutants were unable to induce this redistribution, intranuclear tethering of target receptors may represent yet another, previously uncovered, aspect of SHP inhibition.

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MATERIALS AND METHODS

Plasmids—All plasmids were generated using standard cloning procedures and verified by DNA sequencing. The point mutations in pSG5rSHPmt1 and rSHPmt2 were introduced into the SHP sequence by PCR-mediated mutagenesis using primers containing the mutation, and the inserts were cloned into the EcoRI/BamHI site of pSG5. The PCR-generated fragment of rSHP/H9004H12 (amino acids 1–245) was cloned into the EcoRI site of pSG5. pSG5rSHPwt and pSG5rSHPmt1.2 have been described previously (8). To generate pSG5rHNF-4, HNF-4 was recloned from pLEN4SrHNF-4 (24) into the BamHI site of pSG5. GFP-grGR (GFP fused to the N terminus of GR) was constructed by inserting a BamHI fragment of human GR (amino acids 1–777) into the BglII site of pEGFPC2 (Clontech Laboratories, Inc.) The PGC1A-pSV-Sport (25), pcmv5GR, and the MMTV-Luc reporter plasmid (26) have been described previously. pGL3rPEPCK-Luc (–489 to 73) was generously provided by Dr. Joerg Leers.

Mammalian Cell Transfections—293 human embryo kidney cells and COS-7 monkey kidney cells were maintained as previously described (7, 27). FaoII rat liver hepatoma cells were maintained in RPMI medium supplemented with 10% calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). Transfections were performed using Lipofectin (Invitrogen) as previously described (7), using phenol-free medium for 293 cells in the absence or presence of 1 μM dexamethasone. 24 h after transfection, cells were harvested and luciferase activities were measured. For COS-7 cells, transfections were performed using 0.5 μg of MMTV-Luc reporter, 1 ng (PGC-1 study) or 10 ng of pcmv5GR together with noted amounts of pSG5SHP expression plasmid. For 293 cells, 0.1 μg of pcmv5GR and 0.5 μg of MMTV-Luc reporter was used, together with 0.5 μg of pSG5SHPwt, pSG5SHPmt1, pSG5SHPmt2, pSG5SHPmt1.2 or pSG5SHPH12. For FaoII cells 0.1 μg of PEPCK-Luc reporter was used, together with 10 ng pcmv5GR, 0.1 μg of pSG5HNF-4, and 0.5 μg of pSG5SHPwt, pSG5SHPmt1.2, or pSG5SHPH12. All transfections were performed in 35-mm-diameter plates, and pSG5 (empty vector) was added to equalize total transfected plasmid DNA concentrations.

Coimmunoprecipitation—COS-7 cells were transfected with the corresponding plasmids using DEAE dextran (Amersham Biosciences) in 150-mm-diameter plates and kept for 30 h in the presence of 1 μM dexamethasone. After 30 h, whole cell extracts were prepared using a high-salt buffer containing 10 mM HEPES-KOH, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 5% glycerol, and 1× protease inhibitor mixture (Roche Molecular Biochemicals). Protein extracts were incubated for 2–3 h at

![Fig. 1](image1.png)

**Fig. 1.** Analysis of the inhibitory mechanism of SHP on GR transcriptional activity. A, a schematic representation of the different SHP mutants. B, COS-7 cells were cotransfected with the MMTV-Luc reporter and the expression vector for GR together with 10, 100, or 500 ng of SHP expression plasmid. C, Western blot showing the expression of the different SHP mutants. D, 293 cells were cotransfected with the MMTV-Luc reporter, the expression vector for GR together with expression plasmids for either of the different SHP mutants. Values shown are the means of at least three independent experiments. E, COS-7 cells were transfected with either wild-type SHP, full-length GR, or simultaneously with both constructs. Whole cell extracts were prepared and subjected to immunoprecipitation. After incubation, the samples were analyzed on a Western blot. The SHP protein was detected using a purified polyclonal SHP antibody, and the GR protein was detected using a GR-specific polyclonal antibody, sc-1003. The input represents ~10% of the whole cell extract cotransfected with both SHP and GR.

![Fig. 2](image2.png)

**Fig. 2.** Subcellular distribution of the wild-type and mutated forms of SHP. Expression vectors for the wild-type SHP or for either of the SHP mutants were transiently transfected into COS-7 cells. 8 h posttransfection, the cells were fixed and stained with a purified polyclonal SHP antibody and visualized by confocal microscopy (see “Materials and Methods”). B, table of the subcellular distribution of the different SHP constructs (in percentages).
SHP antibody (1:1000) and the GR-specific polyclonal antibody for 1 h at room temperature. After washing, cells were treated with Lissamine Rhodamine-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS/Tween for 1 h at room temperature, and incubated with purified polyclonal SHP antibody. As seen in Fig. 2, the different SHP proteins appear to have different subcellular distributions. While the existence of localization signals in many cases explains the subcellular distribution of proteins, no conserved localization signals have been identified in SHP, although previous reports revealed a nuclear distribution of a GFP-SHP fusion protein (7). Recent publications have shown a connection between nuclear localization and protein function for different cofactors, including the corepressors SMRT (30) and RIP140 (31). To elucidate the mechanism behind the inability of the different SHP mutants to inhibit GR activity, we decided to study their intracellular localization in mammalian cells. While the existence of localization signals in many cases explains the subcellular distribution of proteins, no conserved localization signals have been identified in SHP, although previous reports revealed a nuclear distribution of a GFP-SHP fusion protein (7). Recent publications have shown a connection between nuclear localization and protein function for different cofactors, including the corepressors SMRT (30) and RIP140 (31). To elucidate the localization of SHP, COS-7 cells were transiently transfected with the different SHP constructs, and 8 h posttransfection, the cells were fixed and stained with a purified polyclonal SHP antibody. As seen in Fig. 2, the different SHP proteins appear to have different subcellular distributions. Wild-type SHP was found predominantly in the nucleus showing a distinct punctuate distribution. Similar, but presumably not identical, nuclear dot patterns have been reported not only for additional corepressors including RIP140 (31) and SMRT (30) but also for the coactivator GRIP-1 (32). The SHP typical dot pattern was found in over 80% of the cells examined, and the remaining 20% of the cells showed either exclusive cytoplasmatic staining or a mixture of both nuclear and cytoplasmatic staining (data not shown). No staining was visible in non-transfected cells. As shown in Fig. 2A, the SHPmt1.2 mutant was also predominantly (74%) localized to the nucleus, showing the same punctuate pattern as the wild-type SHP.

**RESULTS**

**SHP Inhibits the Transcriptional Activity of GR**—Previous studies have demonstrated that SHP functions as a potent inhibitor of various nuclear receptors in mammalian cells. We therefore tested whether SHP could affect GR transcriptional activity under transient transfection conditions. We also used a variety of SHP mutations as tools to dissect the inhibitory mechanism (Fig. 1A), both to investigate the necessity of the previously described LXXLL-related motifs within SHP and also to study the active repression mechanism by using a novel SHP mutation, SHP4H12. This mutant was made in view of previous reports showing that in RXR a deletion of helix 12 promotes the inhibition via stabilization of corepressor interactions (28, 29).

COS-7 cells were cotransfected with the MMTV-Luc reporter and full-length GR, together with increasing amounts of SHP. As shown in Fig. 1B, SHP inhibits dexamethasone-induced GR activity in a dose-dependent manner, leading to an almost complete inhibition. Similar results were observed with human SHP (data not shown). The different SHP mutants were cotransfected into 293 cells, together with the MMTV-Luc reporter and full-length GR. A control Western blot showed that the different SHP proteins were expressed at the same level (Fig. 1C). As shown in Fig. 1D, wild-type SHP inhibits GR activity, as previously seen in COS-7 cells. Interestingly, inhibition of GR activity seems to depend on only one functional NR-box, since mutation of box 1 did not influence the inhibition while mutation of box 2 completely disrupted the ability of SHP to inhibit GR. Not surprisingly, the double NR-box mutant, mt1.2, also lacked the ability to inhibit GR activity. In contrast to the effect of helix 12 deletion on RXR repression (28, 29), deletion of helix 12 in SHP disrupted the inhibition ability, confirming a difference between SHP inhibitory mechanisms compared with other repressing receptors. Furthermore, SHP inhibition of GR was unaffected by trichostatin A, a specific histone deacetylase inhibitor (data not shown), consistent with previous observations (11). To elucidate the interaction between SHP and GR, commounprecipitations were performed using COS-7 cells transfected with GR in the absence or presence of wild-type SHP. Proteins were subjected to immunoprecipitation using a GR-specific mouse monoclonal antibody. As shown in Fig. 1E, lane 2, GR was able to precipitate SHP in the presence of dexamethasone. In addition, GST-pulldowns using GST-SHP protein together with in vitro translated GR, showed a direct interaction between SHP and GR (data not shown).

From these results we conclude that SHP can act as a potent inhibitor of GR activity. Furthermore, both a functional box 2 and an intact helix 12 of SHP appear to be necessary for inhibition of GR.

**Subcellular Distribution of Wild-type and Mutated Forms of SHP**—To further investigate the mechanism behind the inability of the different SHP mutants to inhibit GR activity, we decided to study their intracellular localization in mammalian cells. While the existence of localization signals in many cases explains the subcellular distribution of proteins, no conserved localization signals have been identified in SHP, although previous results revealed a nuclear distribution of a GFP-SHP fusion protein (7). Recent publications have shown a connection between nuclear localization and protein function for different cofactors, including the corepressors SMRT (30) and RIP140 (31). To elucidate the localization of SHP, COS-7 cells were transiently transfected with the different SHP constructs, and 8 h posttransfection, the cells were fixed and stained with a purified polyclonal SHP antibody. As seen in Fig. 2, the different SHP proteins appear to have different subcellular distributions. Wild-type SHP was found predominantly in the nucleus showing a distinct punctuate distribution. Similar, but presumably not identical, nuclear dot patterns have been reported not only for additional corepressors including RIP140 (31) and SMRT (30) but also for the coactivator GRIP-1 (32). The SHP typical dot pattern was found in over 80% of the cells examined, and the remaining 20% of the cells showed either exclusive cytoplasmatic staining or a mixture of both nuclear and cytoplasmatic staining (data not shown). No staining was visible in non-transfected cells. As shown in Fig. 2A, the SHPmt1.2 mutant was also predominantly (74%) localized to the nucleus, showing the same punctuate pattern as the wild-type SHP.
This indicates that nuclear localization, including the dot formation, is independent of nuclear receptor binding and that nuclear localization alone is not sufficient for the inhibitory effect. In comparison, the SHPΔH12 mutant was exclusively localized in the cytoplasm, either unable to localize to the nucleus or actively exported out of the nucleus.

Redistribution of GR within Nuclear Compartments by Wild-type SHP but Not by the Inhibition-deficient SHP Mutants—The distinct distribution pattern of both SHP (Fig. 2) and GR (33, 34) enabled us to investigate the combined effect of both proteins regarding their cellular distribution. To study the localization of GR, a GFP-GR construct was prepared, using the full-length human GR. This construct enables transcriptional activation in transient transfections using a GRE-Luc reporter in the presence of dexamethasone (data not shown). GFP-GR was transfected into COS-7 cells, and 5 h posttransfection dexamethasone was added to selected wells. The cells were left for an additional 3 h, fixed, and visualized in the confocal microscope. As previously reported (33, 34), GFP-GR, in the absence of ligand, is uniformly distributed in the cytoplasm (Fig. 3Aa) but is translocated into the nucleus in the presence of dexamethasone (Fig. 3Ab). To determine the effect of SHP on GFP-GR distribution, COS-7 cells were cotransfected with GFP-GR together with wild-type SHP, and the cells were treated as described above. After fixation, the cells were stained with a purified polyclonal SHP antibody and visualized in the confocal microscope. No effect of the antibody-staining procedure was seen on cells only expressing GFP-GR (data not shown). In cells containing both GFP-GR and SHP, in the absence of dexamethasone, both proteins showed their typical individual pattern, see Fig. 3B, a and b. This is consistent with the idea that SHP only interacts with ligand-activated GR. Addition of dexamethasone leads to a relocation of GFP-GR into the nucleus as expected, but the presence of SHP seems to redistribute GFP-GR from the rather diffuse nuclear distribution to a specific dot pattern, which was never observed in the absence of SHP, see Fig. 3Bd. The dot pattern of GFP-GR shows an overlapping distribution with the dot pattern of SHP (Fig. 3Bf), consistent with a possible physical in vivo interaction between the two proteins. Apparently, SHP distribution determines the localization of the GR/SHP complex in the presence of dexamethasone. The colocalization of SHP and GR was seen in ~64% of the cotransfected cells. In the remaining cells, the same dot pattern was seen for GR, but SHP was instead localized to the cytoplasm. If the redistribution of GR into the specific dot pattern seen in Fig. 3B, d–f is associated with inhibition of transcription, then a SHP mutant lacking the inhibition ability may not affect the distribution of GR to the same extent as wild-type SHP. Cotransfections were performed using the GFP-GR construct together with either of the SHP mutants, and the cells were treated as in the former experiment. As shown in Fig. 3, C and D, none of the repression-deficient mutants was able to affect the GFP-GR distribution. Instead, GFP-GR in the presence of dexamethasone was localized to the nucleus into its characteristic diffuse pattern, and the different SHP mutants showed their original pattern. In the absence of dexamethasone, GFP-GR is distributed all over the cytoplasm, with no obvious effect of coexpression with the different SHP mutants (data not shown). These results indicate (i) that functional NR-boxes are necessary for the relocation of GR since the SHPmt1.2 despite its punctuate pattern is unable to induce relocation of GR, and (ii) that loss of nuclear distribution of the SHPΔH12 mutant affects the ability to inhibit GR function. In conclusion, we suggest that the intranuclear redistribution of ligand-activated GR by SHP could be linked to inhibitory mechanisms and that the inability of the SHP mutants to alter the intracellular localization of GR could be a reason for their inability to inhibit GR activity.

SHP Antagonizes PGC-1 Coactivation of GR in Mammalian Cells—As shown in Fig. 1, a functional NR-box 2 of SHP seems to be necessary for inhibition of GR. Intriguingly, when searching databases for SHP box 2-homologous sequences, the closest motif in any mammalian protein found was the NR-box in the nuclear receptor coactivator PGC-1 (Fig. 4A). PGC-1 was first identified
as a coactivator for PPARγ and thyroid receptor (25), linked to adaptive thermogenesis. Interestingly, PGC-1 has lately been shown to play an important role in up-regulating gluconeogenesis in liver by acting as a coactivator for GR and HNF-4 (35, 36). PGC-1 mRNA was induced by glucocorticoids and cAMP, by fasting, or in other states of relative insulin deficiency. PGC-1 contains only one NR-box that is involved in the ligand-dependent interaction and coactivation of these receptors. Since SHP box 2 and the PGC-1 motif show such a high homology, we asked whether SHP would antagonize the ability of PGC-1 to potentiate fasting, or in other states of relative insulin deficiency. PGC-1 has lately been shown to play an important role in up-regulating gluconeogenesis by acting as a coactivator for GR and HNF-4 (35, 36). Furthermore, both SHP and PGC-1 motifs fall into the LXXLL peptide class III (40). The ability of SHP to antagonize PGC-1 coactivation indicates competition for binding to GR and thus supports the functional relevance of the LXXLL-related SHP motif 2 for receptor interactions. Similar binding specificity for this intriguing pair of inducible coregulators, the corepressor SHP and the coactivator PGC-1, might give rise to functional antagonism on additional relevant target receptors such as HNF4 (35) and ERα (41) including their regulated genes.

When investigating wild-type SHP and the various inhibition-deficient mutants we observed a clear variation with respect to their subcellular distribution. The reason for this variation is currently unknown as no nuclear export or import signal has been identified in SHP. Perhaps putative SHP corepressors have an important role in maintaining the nuclear localization of wild-type SHP and SHPmt1.2 but not of SHPΔH12 (27). In addition, the distribution of wild-type SHP in both cytoplasm and nucleus could indicate a shuttling potential and reveal a possible, novel regulation mechanism of SHP activity, as previously being described for DAX-1 function (8). When investigating the necessity of the two functional SHP motifs for GR inhibition we could now clearly demonstrate that the closest motif in any mammalian protein is the LXXLL motif of the GR coactivator PGC-1. The homology extends beyond the leucine core to adjacent residues, suggesting similar binding specificity and shared target receptors. Furthermore, both SHP and PGC-1 motifs fall into the LXXLL peptide class III (40). The ability of SHP to antagonize PGC-1 coactivation indicates competition for binding to GR and thus supports the functional relevance of the LXXLL-related SHP motif 2 for receptor interactions. Similar binding specificity for this intriguing pair of inducible coregulators, the corepressor SHP and the coactivator PGC-1, might give rise to functional antagonism on additional relevant target receptors such as HNF4 (35) and ERα (41) including their regulated genes.

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The relocalization of GR in the presence of SHP, from a rather diffuse nuclear distribution to a specific dot pattern that...
overlaps with the SHP dots, indicates a specific in vivo interaction between the two proteins. Notably, a similar phenomenon has been described for RARs, which in the absence of ligand is redistributed from a diffuse nuclear pattern into distinct dots, overlapping with the corepressor SMRT (30). Although the functional relevance of the SMRT-RAR or the SHP-RAR dots is currently unknown, they could possibly be linked to the inhibition mechanism. For example, SHP could tether GR to subnuclear locations where GR would no longer be accessible for the transcriptional machinery. Alternatively, these locations could represent assembly sites for functional corepressor complexes. In addition, because we have observed that a functional LAXXLL-related motif 2 is required for agonist-dependent GR relocalization by SHP in vivo, it is likely that this is due to direct physical interactions between the GR AF-2 and SHP motif 2.

The ability of SHP to inhibit deoxymethasone-induced activation of the PEPCK promoter, in addition to the fact that SHP and GR are coexpressed in liver, argues for a physiological role of SHP in glucocorticoid signaling. Our finding that SHP antagonizes the activity of PGC-1 on physiologically relevant target receptors and target genes suggests a structural and functional relationship between the coactivator PGC-1 in glucose homeostasis. Notably, the in vivo function of SHP in glucocorticoid signaling. Our finding that SHP antagonizes the activity of PGC-1 on physiologically relevant target receptors and target genes suggests a structural and functional relationship between the coactivator PGC-1 in glucose homeostasis.