Molecular characterization of SMILE as a novel corepressor of nuclear receptors

Yuan-Bin Xie, Balachandar Nedumaran and Hueng-Sik Choi*

Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju, 500-757, Republic of Korea

ABSTRACT

SMILE (small heterodimer partner interacting leucine zipper protein) has been identified as a coregulator in ER signaling. In this study, we have examined the effects of SMILE on other NRs (nuclear receptors). SMILE inhibits GR, CAR and HNF4α-mediated transactivation. Knockdown of SMILE gene expression increases the transactivation of the NRs. SMILE interacts with GR, CAR and HNF4α *in vitro* and *in vivo*. SMILE and these NRs colocalize in the nucleus. SMILE binds to the ligand-binding domain or AF2 domain of the NRs. Competitions between SMILE and the coactivators GRIP1 or PGC-1α have been demonstrated *in vitro* and *in vivo*. Furthermore, an intrinsic repressive activity of SMILE is observed in Gal4-fusion system, and the intrinsic repressive domain is mapped to the C-terminus of SMILE, spanning residues 203–354. Moreover, SMILE interacts with specific HDACs (histone deacetylases) and SMILE-mediated repression is released by HDAC inhibitor trichostatin A, in a NR-specific manner. Finally, ChIP (chromatin immunoprecipitation) assays reveal that SMILE associates with the NRs on the target gene promoters. Adenoviral overexpression of SMILE represses GR-, CAR- and HNF4α-mediated target gene expression. Overall, these results suggest that SMILE functions as a novel corepressor of NRs via competition with coactivators and the recruitment of HDACs.

INTRODUCTION

Small heterodimer partner interacting leucine zipper protein (SMILE) belongs to basic region leucine zipper (bZIP) family (1,2). SMILE gene produces two isoforms, SMILE-L (long isoform of SMILE, also known as CREBZF) and SMILE-S (short isoform of SMILE, previously known as Zhangfei), from alternative usage of initiation codons (2). Although SMILE has the ability to homodimerize like other bZIP proteins, it cannot bind to DNA as a homodimer (1–3). SMILE has been identified as an interacting partner of herpes simplex virus-related host-cell factor (HCF) and inhibits the replication of the herpes simplex virus (1,3). SMILE has also been reported as a coactivator of ATF4 and as a corepressor of CREB3, another cellular HCF-binding transcription factor (4,5). Recently, we have reported that SMILE acts as a coregulator of estrogen receptor (ER) signaling (2), but its role in other nuclear receptors (NRs) signaling remains unknown.

NRs are transcription factors that modulate the expression of genes involved in embryonic development, maintenance of differentiated cellular phenotypes, metabolism and cell death [see references (6–8) for reviews]. Members of the NR superfamily include the conventional endocrine receptors, the adopted orphan receptors, for which ligands have been identified in recent years, and the orphan receptors, ligands of which have not yet been identified (8). Glucocorticoid receptor (GR) is a member of steroid receptor family and mediates the effect of glucocorticoids in a variety of cellular processes, including homeostasis, cell growth, development, stress response and inflammation (8). GR regulates the transcription of target genes either by binding to specific glucocorticoid response elements (GREs) within the target genes or by interacting with other DNA-bound transcription factors. The inactive GR resides in the cytoplasm bound to heat-shock protein. It dissociates from heat-shock protein upon ligand binding and enters the nucleus where it functions as a transcription factor (9). GR plays an important role in various metabolic pathways by regulating the expression of genes such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and insulin-like growth factor-binding protein 1 (IGFBP1) (10,11).

Constitutive androstane receptor (CAR) is an adopted orphan NR which functions as heterodimers with the retinoid X receptor (RXR) (8). CAR evidences constitutive activity, and is expressed primarily in the liver, where it

*To whom correspondence should be addressed. Tel: +82 62 530 0503; Fax: +82 62 530 0506. Email: hsc@chonnam.ac.kr

regulates many Phase I and Phase II biotransforming enzymes, including Cyp2b6, Sult2a1, SultN and Ugt1a1 (12,13). This xenobiotic receptor can also regulate the expression of membrane transporter proteins such as organic anion transporting peptide 2 (Oatp2) and multi-drug resistance-associated proteins. CAR can be modulated by structurally diverse chemicals such as 1,4-*bis*-2 [-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and phenobarbital (12–14). Hepatocyte nuclear factor 4 (HNF4) is an orphan nuclear receptor which is highly expressed in the liver, kidney, and pancreatic β-cells. HNF4 contains two subtypes in mammals, namely HNF4α and HNF4γ, and binds to the DR-1 element of target gene promoters as homodimers (6,7). HNF4α plays critical roles not only in the specification of the hepatic phenotype during liver development but also in the transcriptional regulation of genes involved in glucose, cholesterol, fatty acids and xenobiotic metabolism (7, 15), including PEPCK, cholesterol 7 alpha-hydroxylase (CYP7A1) and liver carnitine palmitoyl transferase CPT (L-CPT) (16–18). Mutations in the HNF4α gene have been associated with maturity-onset diabetes of the young (MODY) (7).

NR-mediated transcriptional effects are regulated by NR coregulators, including coactivators and corepressor (19). Coregulators modulate the transcription of NR target genes through taking part in chromatin remodeling or interacting with basal transcriptional machinery to influence the main steps in transcriptional initiation (20). In the presence of NR ligands, the SWI/SNF chromatin remodeling complex, the histone acetyltransferase (HAT) activity containing complexes CBP/p160/P/CAF, and the TRAP/DRIP/ARC complex are sequentially recruited to gene promoters to activate gene transcription (21–24). Coactivators of the p160 family, including SRC1/NCoA1 and TIF-2/GRIP1, interact with the ligand-binding domain (LBD)/activation function 2 (AF2) domain of receptors through an LXXLL motif or NR boxes (25). In the absence of NR ligands, on the other hand, many NRs prevent gene transcription via recruitment of corepressors such as N-CoR and SMRT, which have been proposed to antagonize the actions of coactivators and to maintain a more repressed state in the chromatin structure. Histone deacetylases (HDACs)-dependent and HDACs-independent mechanisms are involved in the transrepression induced by N-CoR and SMRT (26).

In this study, we have identified that SMILE represses the transcriptional activities of GR, CAR and HNF4α through direct interaction. We have demonstrated that SMILE represses the transactivities of the NRs via competition with coactivators and the recruitment of HDACs for its active repression. Overall, our findings suggest that SMILE acts as a novel corepressor of NRs.

MATERIALS AND METHODS

Plasmid and DNA construction

The plasmids of pCMV-β-gal, pcDNA3mCAR, pcDNA3 mCARΔAF2 and pcDNA3-HA-mPPARγ, -PGC-1α, pSG5HA-GRIP1, (NR1)X5-Luc, Gal4-tk-Luc and PPRE-Luc were described elsewhere (27–29).

p(HNF4)8-tk-Luc and MMTV-Luc were kindly provided by Drs Akiyoshi Fukamizu and Yoon-Kwang Lee, respectively. pcDNA3-SMILE, pcDNA3-Flag-SMLE, pcDNA3-SMILE-83Leu, pcDNA3-SMILE-1Phe, pGEX4T-1, pGEX4T-1-SMILE, pEBG, pEGFP-SMILE, pSuper, pSuper-siSHP, pSuper-siSMILE-I and pSuper-siSMILE-II were described previously (2).

pcDNA3-Flag-mCAR, pcDNA3-HA-mCAR and pcDNA3-HA-HNF4α were constructed by inserting the full PCR fragments of the ORFs into the EcoRI/XhoI sites of pcDNA3-Flag, or pcDNA3-HA vector. pcDNA3-HA-mGR was generated by subcloning the full ORF of mouse GR into the XhoI/XbaI sites of pcDNA3-HA vector. Mouse GR deletion constructs, including pcDAN3-mGR-N (1–531 aa) and pcDNA3-mGR-LBD (532–783 aa), were subcloned via the insertion of the PCR fragments of mouse GR into pcDNA3 between the BamHI and XhoI sites. The pcDNA3-HA-HNF4αCD (1–370 aa) and pcDNA3-HA-HNF4αΔLBD (1–174 aa) plasmids were constructed via subcloning the EcoRI-XhoI cDNA fragments of rat HNF4α into pcDNA3-HA vector. The SMILE leucine zipper region mutant SMILE-L (239–267)V was generated via PCR-mediated site-directed mutagenesis, and the PCR products were cloned into the EcoRI/XhoI sites of pcDNA3-Flag and the BamHI/KpnI sites of pEBG vector. pEBG-SMILE and pEBG-SMILE deletion constructs were constructed by inserting full length SMILE or appropriate SMILE deletion fragments into pEBG vector between BamHI and KpnI sites. All plasmids were confirmed via sequencing analysis.

Gal4-DBD fusion constructs were generated using the pCMX-Gal4N expression vector (30). To generate Gal4-DBD-SMILE, EcoRI/XhoI digested full-length SMILE fragments from pcDNA3-Flag-SMILE were cloned into EcoRI/SalI-digested pCMX-Gal4N vector. To construct the Gal4-DBD-SMILE deletion constructs, SMILE cDNA deletion fragments were obtained from pcDNA3-Flag-SMILE via PCR, and cloned into pCMX-Gal4N vector between the EcoRI and SalI sites. pSuper-siHDAC1, pSuper-siHDAC3 and pSuper-siHDAC4 constructs were constructed by inserting a 64-bp double-stranded oligonucleotide containing 5'-aagcaga tgcagagattcaac-3' of the human HDAC1 cDNA sequence, or 5'-aagatgctgaaccatgcacct-3' of human HDAC3 cDNA sequence, or 5'-aatgtacgacgccaaagat-3' of human HDAC4 cDNA sequence into the pSUPER vector between BglII and Xho I sites. All plasmids were confirmed via sequencing analysis.

Cell culture, transient transfection assay and luciferase assay

HEK293T (293T), HepG2 and HeLa cells were obtained from the American-type culture collection (ATCC) and cultured according to the manufacturer's instructions. Transient transfection was performed using Superfect transfection reagent (Qiagen) as described previously (2). Total amounts of DNA in each transfection were maintained at the same levels using empty pcDNA3 vectors. Luciferase assays were performed as described

previously (31). Fold activity was calculated considering the activity of reporter gene alone as 1.

In vitro glutathione S-transferase (GST) pull-down assay and competition assay

In vitro GST pull-down and competition assays were performed as described previously (31,32).

Co-immunoprecipitation (Co-IP) and western blot analysis

Co-IP and western blot analysis were performed as described previously (2,33). Antibodies used for Co-IPs were anti-GR (Santa Cruz, sc-8992), anti-CAR (Santa Cruz, sc-13065) and anti-HNF4α antibody (Santa Cruz, sc-8987). Control Co-IPs were carried out using rabbit IgG (Santa Cruz, sc-2027). In western blot assays, the following antibodies were used at dilution of 1:1000: anti-Flag M2 (Stratagene, #200472-21), anti-HA (12CA5) (Roche Molecular Biochemicals), anti-SMILE (Abcom, #ab28700), anti-GST (Santa Cruz, sc-33614) and anti-tubulin (Cell Signaling Technology, #2146) antibodies. In western blot analysis of immunoprecipitated proteins, conventional HRP conjugated anti-rabbit IgG was replaced with rabbit IgG TrueBlot (eBioscience, #18-8816) to eliminate signal interference by the immunoglobulin heavy and light chains.

In vivo GST pull-down assay

In vivo GST pull-down experiments were performed as described previously (2). In brief, 293T cells were transfected in 60 mm dishes with the indicated plasmids. Forty-eight hours after transfection, the whole-cell extracts were prepared and equal amounts of total protein were used for *in vivo* GST pull-down assays followed by western blot analysis.

Confocal microscopy

The confocal microscopy assays were carried out as described previously (2). In brief, the HeLa cells grown on gelatin-coated coverslips were transfected with indicated plasmids using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Twelve hours after transfection, the cells were treated with or without 100 nM dexamethasone for 12 h followed by cell fixation and immunostaining. To detect HA-fusion proteins and nucleus, the cells were incubated with dye Alexa 594-conjugated anti-HA monoclonal antibody (1:500 dilution; Invitrogen) for 1 h at room temperature (25°C), washed three times in PBS, and incubated with 0.1 mg/ml of DAPI (Invitrogen) solution for 10 min. After three times washing with PBS, the cells were subjected to observation by confocal microscopy.

Preparation of recombinant adenovirus

The adenovirus-encoding human SMILE was described previously (2). The adenovirus-encoding rat HNF4α was constructed via the previously described method (2). In brief, the cDNA-encoding rat HNF4α was cloned into the KpnI/XbaI sites of the pAdTrack-CMV vector. The recombination of the AdTrack-CMV-rHNF4α (where rHNF4α is rat HNF4α) with adenoviral gene carrier vector was performed by transformation to pre-transformed adEasy-BJ21-competent cells.

RNA interference

Knockdown of SMILE and histone deacetylases (HDACs) was performed using the pSuper vector system (2). 293T cells were transfected with siRNA constructs using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. siRNA-treated cells were subjected to reverse transcription-PCR (RT–PCR) or the second transfection as indicated in the figure legends.

RT–PCR analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The mRNAs of SMILE, SHP, insulin-like growth factor-binding protein 1 (IGFBP1), CYP2B6 and cholesterol 7α-hydroxylase (CYP 7A1) were analyzed by RT–PCR as previously described (2), and the mRNA levels of β-actin served as an internal control for RT–PCR. The RT–PCR primers are provided in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP) Assay

ChIP assay was performed as previously described (32). In brief, HepG2 cells seeded into 35 mm culture dishes were treated as indicated in the figure legends and then the cells were fixed with 1% formaldehyde, washed with ice-cold PBS, harvested and sonicated. The soluble chromatin was then subjected to immunoprecipitation using anti-GR, anti-CAR, anti-HNF4α, anti-SMILE (Santa Cruz, sc-49329), or acetyl-histone H3 (Lys9) antibody (Cell Signaling Technology, #9671) followed by protein A agarose/salmon sperm DNA (Upstate). DNA was recovered via phenol/chloroform extraction and amplified by PCR for 30–35 cycles using specific primer sets for the indicated specific regions of IGFBP1, CYP2B6 and CYP7A1 genes. The PCR primers for ChIP assay are provided in Supplementary Table 2.

Statistical analysis

Student's *t*-test was performed using GraphPad Prism version 3.0 for Windows and results were considered to be statistically significant when $P < 0.05$.

RESULTS

SMILE inhibits the transactivation of nuclear receptor GR, CAR and HNF4α

Previously, we have reported that SMILE regulates orphan NR small heterodimer partner (SHP)-repressed ER transactivation through direct interaction with SHP (2). To investigate whether SMILE interacts with other NRs, yeast two-hybrid interaction assays were performed. Of great interest, SMILE was determined to interact with many NRs, including GR, TRα, CAR, SF-1, ERRα, ERRβ, ERRγ, HNF4α and Nur77 (Supplementary Table 3). For detailed study, GR, CAR and HNF4α
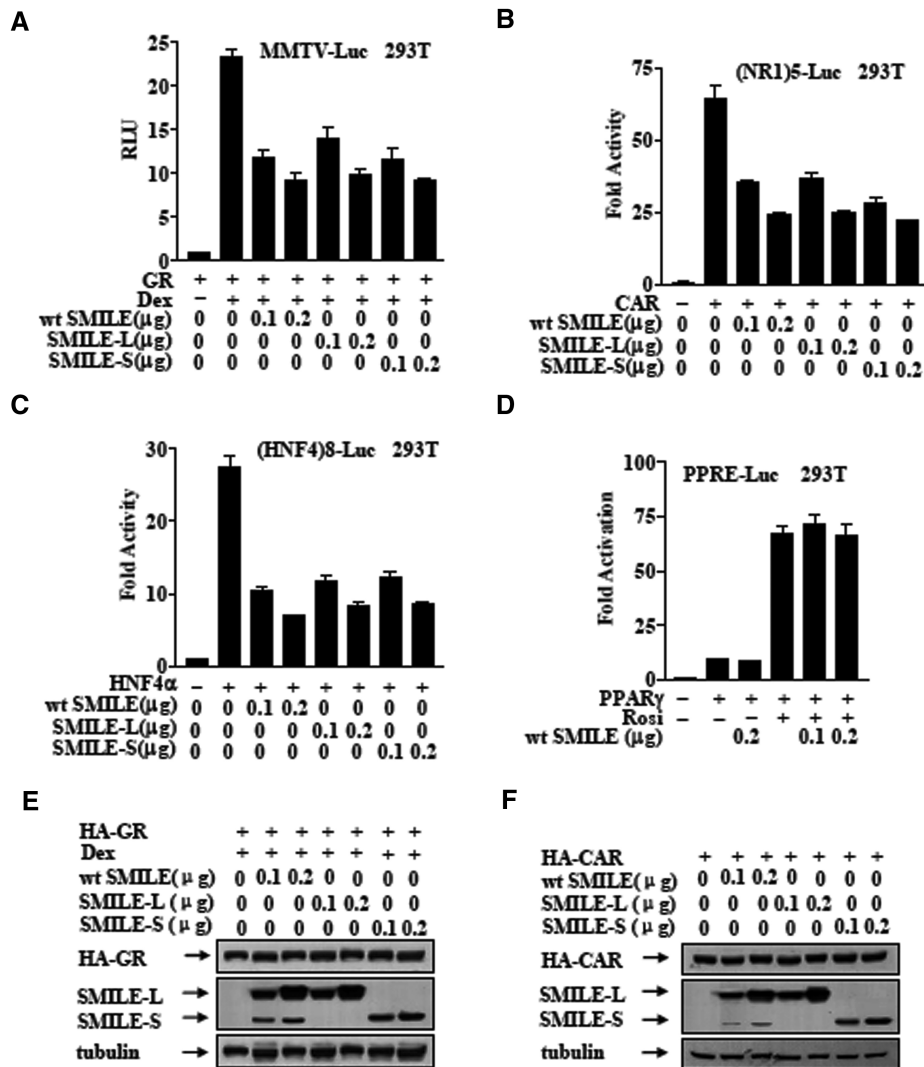
Figure 1. SMILE represses GR-, CAR- and HNF4α transactivation. Reporter assays (**A–D**) were performed as described in the Materials and methods section. HEK293T (293T) cells were cotransfected with 0.1 μg of pcDNA3-HA-GR (A), pcDNA3-HA-CAR (B), pcDNA3-HA-HNF4α (C) or, pcDNA3-HA-PPARγ (D) and 0.1 μg of MMTV-Luc (A), (NR1)5-Luc (B), (HNF4)8-Luc (C) or, PPRE-Luc (D) luciferase reporter vectors, 0.1 μg of pCMV-β-gal as internal control, together with indicated amounts of plasmids expressing wild-type SMILE, SMILE-L (SMILE-83Leu) and SMILE-S (SMILE-1Phe). Twenty-four hours after transfection, the cells were treated with or without 100 nM of dexamethasone (Dex) (A) or rosiglitazone (Rosi) (D) as indicated for 24 h prior to the measurement luciferase activity. The effects of overexpressed SMILE on the protein levels of HA-GR (**E**) and HA-CAR (**F**). 293T cells were cotransfected with various plasmids as indicated. Fifty microgram of cellular extracts from the transient transfection assay were subjected to western blot analysis. The proteins of HA-GR, HA-CAR, SMILE and tubulin were detected as described in the Materials and methods section. wt, wild-type.

were selected as a representative of classical endocrine receptors, adopted orphan receptors, and orphan receptors (8), respectively.

We have previously demonstrated that wild-type SMILE generates two isoforms, SMILE long form (SMILE-L) and SMILE short form (SMILE-S), which can be produced by the mutants SMILE-83Leu and SMILE-1Phe, respectively (2). To determine whether these isoforms can regulate the transactivation of GR, CAR and HNF4α, transient transfection was performed in 293T cells. Overexpression of wild-type SMILE dose-dependently repressed dexamethasone-stimulated GR transactivation (Figure 1A), as well as CAR and HNF4α transactivation (Figure 1B and C). Furthermore, overexpression of SMILE-L or SMILE-S through

the aforementioned SMILE mutants evidenced similar inhibitory effects on the NRs (Figure 1A–C). However, SMILE did not show any significant effect on PPARγ-mediated transactivation (Figure 1D). Western blot analysis demonstrated that the overexpression of wild-type SMILE, SMILE-L or SMILE-S alone did not significantly change the protein expression of HA-GR and HA-CAR (Figure 1E and F). Taken together, these results suggest that both SMILE isoforms down-regulate GR, CAR and HNF4α transactivation. Since SMILE-L and SMILE-S show similar effects on those NRs, and SMILE-L is the major isoform in tested cell lines and tissues (2), we have focused on wild-type SMILE-generated SMILE-L (SMILE) for further investigations.

A

B

C

D
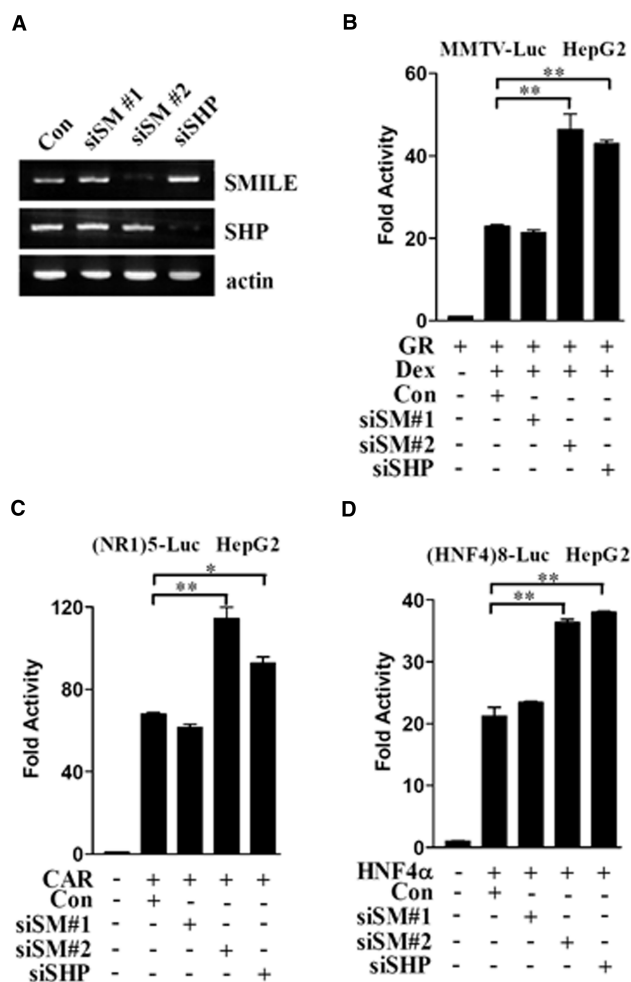
Figure 2. siSMILE increases GR-, CAR- and HNF4α transactivation. (**A**) Effect of siRNAs for SMILE or SHP on the expression of SMILE and SHP. HepG2 cells were transfected with pSUPER siSMILE-I (siSM#1), siSMILE-II (siSM#2), siSHP or pSUPER [control (con)], and after 72 h the total RNA was isolated. The mRNA levels of SMILE and SHP were measured via RT–PCR analysis, with β-actin shown as a control. The data shown are representative of at least three independent experiments. (**B–D**) SMILE siRNA induces GR-, CAR- and HNF4α-mediated transactivation in HepG2 cells. HepG2 cells were transfected with pSUPER, pSUPER siSMILE-I (siSM#1), or pSUPER siSMILE-II (siSM#2). After 24 h, the cells were cotransfected with expression vector for GR (B), CAR (C) or HNF4α (D), and MMTV-Luc (B), (NR1)5-Luc (C) or (HNF4)8-Luc (D) luciferase reporter vectors, together with pCMV-β-gal as internal control. Twenty-four hours after transfection, the cells in (B) were treated for 24 h with or without 100 nM Dex prior to the measurement of luciferase activity. The mean and standard deviation ($n = 3$) of a representative experiment are shown. $^{*}P < 0.05$; $^{**}P < 0.01$, using Student's *t*-test.

## Knockdown of SMILE gene expression up-regulates GR, CAR and HNF4α transactivation

To determine whether endogenous SMILE negatively regulates GR, CAR and HNF4α transactivation, we investigated these NRs-mediated transcriptional activities after individually knocking down the expression of SMILE and SHP in HepG2 cells. As shown in Figure 2A, siSMILE-II (siSM-II) efficiently knocked down the mRNA levels of SMILE, whereas siSMILE-I (siSM-I) did not show any

significant effect, and siSHP efficiently silenced the gene expression of SHP, which is a well-known corepressor of GR, CAR and HNF4α (2). In the reporter assay, knockdown of SMILE gene expression through siSMILE-II increased GR-, CAR- and HNF4α-mediated transactivation by 65–100%, which is similar to the effect of positive control siSHP (Figure 2B–D). Collectively, these results indicate that endogenous SMILE is a functional corepressor of receptor GR, CAR and HNF4α.

## SMILE interacts with GR, CAR and HNF4α *in vitro* and *in vivo*

To determine whether SMILE inhibits GR, CAR and HNF4α transactivation through protein–protein interaction, *in vitro* and *in vivo* GST pull-down experiments were performed. For the *in vitro* GST pull-down assays, bacteria-expressed GST only, or GST-SMILE proteins were incubated with *in vitro* translated ^{35}S-labeled GR, CAR, or HNF4α. We found that ^{35}S-labeled GR was able to bind to GST-tagged SMILE in the presence of dexamethasone, but not in the absence of the ligand (Figure 3G, upper panel), indicating that SMILE can interact with GR in a ligand-dependent manner. Moreover, ^{35}S-labeled CAR, and HNF4α were also observed to bind to GST-SMILE (Figure 3G, lower panel). These results suggest that SMILE can interact with GR, CAR and HNF4α *in vitro*. For the *in vivo* GST pull-down assays, mammalian expression vectors encoding either pEBG (GST) alone or pEBG-SMILE (GST-SMILE) together with indicated pcDNA3-HA-GR, pcDNA-Flag-mCAR, or pcDNA3-HA-HNF4α were cotransfected into 293T cells. As shown in Figure 3A, HA-GR was detected in the coprecipitate only in the presence of its ligand when coexpressed with GST-SMILE but not with GST alone. The expression levels of GST, GST-SMILE and HA-GR were confirmed by western blot analysis (Figure 3A, middle and bottom panels, respectively). These results demonstrate that ectopically expressed SMILE interacts with exogenous GR in a ligand-dependent manner in 293T cells. Similarly, interactions of exogenous SMILE with CAR (Figure 3B) and HNF4α (Figure 3C) were verified using *in vivo* GST pull-down assays. To further examine whether endogenous SMILE and these NRs can interact *in vivo*, co-immunoprecipitation assays were performed. Endogenous SMILE proteins were found to be co-precipitated with GR in a ligand-dependent manner (Figure 3D), while with CAR in a ligand-independent manner (Figure 3E). In addition, endogenous SMILE was co-precipitated with endogenous HNF4α (Figure 3F), confirming the interaction between that endogenous SMILE and the NRs. Collectively, these results indicate that SMILE can interact with GR, CAR and HNF4α both *in vitro* and *in vivo*.

To examine whether SMILE and its binding partners (GR, CAR, or HNF4α) are colocalized to the same subcellular compartments, confocal microscopic studies were performed. HeLa cells were cotransfected with the expression plasmids pEGFP-SMILE along with pcDNA3-HA-GR, or pcDNA3-HA-mCAR, or pcDNA3-HA-HNF4α, stained with dye Alexa 594-conjugated anti-HA antibody
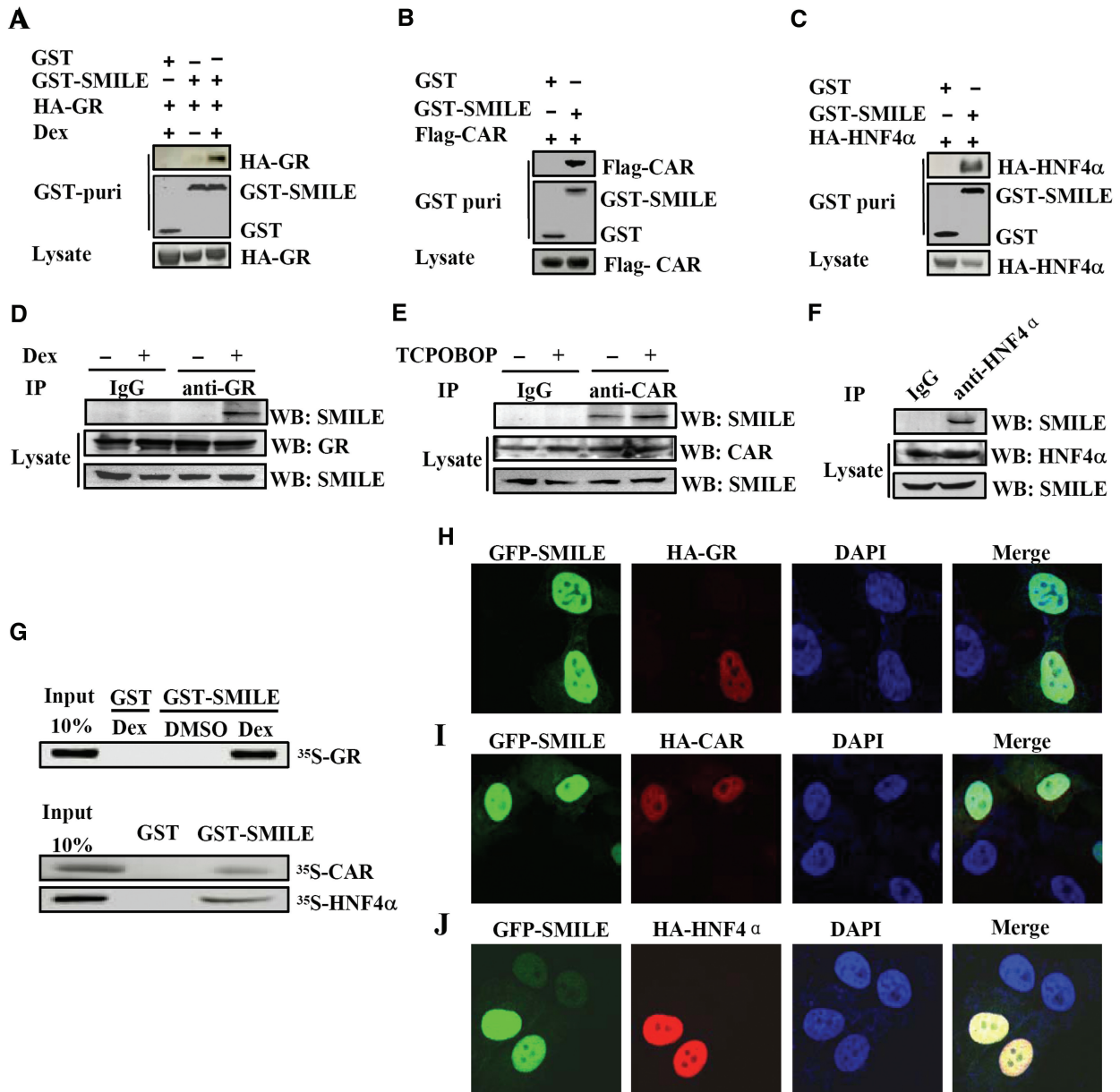
Figure 3. Interactions and colocalizations of SMILE with NRs. (**A–C**) *In vivo* interactions of exogenous GR (A), CAR(B) and HNF4α (C) with exogenous SMILE. 293T cells were cotransfected with expression vectors for HA-GR (A), Flag-mCAR (B), or HA-HNF4α (C) with pEBG-SMILE (GST-SMILE) or pEBG alone (GST). The *in vivo* GST pull-down assays were performed in the presence or absence of the GR ligand Dex (100 nM) as indicated (A). The complex formation (top panel in A–C, GST puri.) and the amount of HA-GR, Flag-mCAR or HA-HNF4α used for the *in vivo* binding assay (bottom panel in A–C, Lysate) were determined via western blot using an anti-HA or anti-Flag antibody. The same blot was stripped and reprobed with an anti-GST antibody (middle panel in A–C) to confirm the expression levels of the GST fusion protein (GST-SMILE) and the GST control (GST). *In vivo* interactions of endogenous GR (**D**), CAR (**E**) and HNF4α (**F**) with endogenous SMILE. Co-immunoprecipitation assays were performed using cell extract from HepG2 cells using indicated antibodies in the presence or absence of 100 nM Dex or 250 nM of TCPOBOP. Endogenous SMILE was immunoprecipitated with GR, CAR and HNF4α (upper panels). The proteins in the cell lysates (middle and lower panels) were analyzed with western blot analysis using indicated antibodies. (**G**) *In vitro* GST pull-down assays. Upper panel, ^{35}S-radiolabeled GR protein was incubated with GST, or GST-SMILE fusion proteins in the presence of 100 nM Dex or vehicle (DMSO). Lower panel: ^{35}S-radiolabeled HNF4α, or CAR proteins were incubated with GST, or GST-SMILE fusion proteins. The input lane represents 10% of the total volume of *in vitro*-translated proteins used for binding assay. Protein interactions were detected via autoradiography. (**H–J**) Co-localizations of SMILE with NRs. Hela cells grown on coverslips on 12-well plates were transfected with 0.1 μg of expression vectors encoding GFP-SMILE and HA-GR (H), HA-CAR (I) or HA-HNF4α (J). Twelve hours after transfection, the cells (H) were treated with 100 nM Dex for 12 h. For the immunofluorescence of fixed cells, the HA-fusion proteins were detected with dye Alexa 594-conjugated anti-HA monoclonal antibody. The cell images were captured under 400 × magnifications. The data shown are representative of at least three independent experiments.
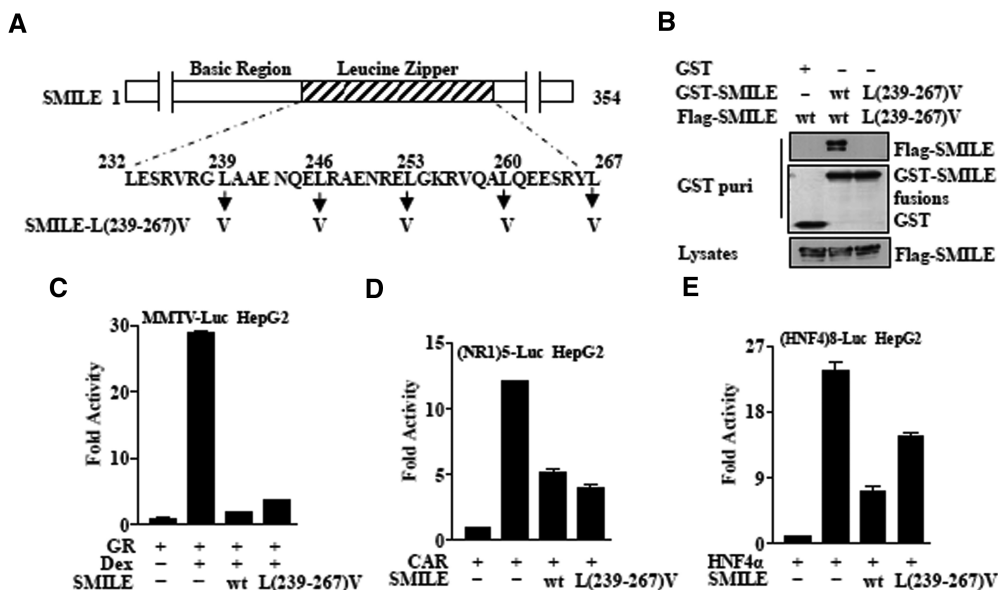
Figure 4. Homodimerization of SMILE is not essential for the repressive function. (**A**) Structure of human SMILE. The basic region and leucine zipper domains are shown. The leucine zipper mutant SMILE-L (239–267)V indicates the leucine residues between positions 239 and 267 were mutated to valine, as indicated by the arrows. The numbers in the figure indicate the amino-acid residues. (**B**) *In vivo* homodimerization possibility analysis of wt SMILE and SMILE-L (239–267)V. 293T cells were cotransfected with expression vectors for Flag-SMILE, Flag-SMILE-L (239–267)V with pEBG-SMILE (GST-SMILE wt) or pEBG-SMILE-L (239–267)V or pEBG alone (GST) as indicated. The complex formation (top panel, GST puri.) and the amount of Flag-SMILE, Flag-SMILE-L (239–267)V used for the *in vivo*-binding assay (bottom panel, Lysate) were determined by western blot using anti-Flag antibody. The same blot was stripped and reprobed with an anti-GST antibody (middle panel) to verify the expression levels of the GST fusion proteins (GST-SMILE) and the GST control (GST). (**C–E**) The effect of SMILE-L (239–267)V on GR, CAR and HNF4α-mediated transactivation. HepG2 cells were cotransfected with expression vector for GR (C), CAR (D), or HNF4α (E) and MMTV-Luc (C), (NR1)5-Luc (D), or (HNF4)8-Luc (E) luciferase reporter vectors, pCMV-β-gal as internal control, together with expression vectors for wt SMILE or SMILE-L (239–267)V as indicated. Twenty-four hours after transfection, the cells in (C) were treated with 100 nM Dex for 24 h. Forty-eight hours after tranfection, luciferase activity was measured. wt, wild type. The mean and standard deviation (*n* = 3) of a representative experiment are shown.

and DAPI, and analyzed via confocal microscopy. As shown in Figure 3H, GFP-SMILE was predominantly localized within the nucleus, and was also weakly detected in the cytoplasm, which was consistent with the results of our previous study (2). In the presence of ligand, GR was detected predominantly in the nucleus (Figure 3H). CAR (Figure 3I) and HNF4α (Figure 3J) were also detected mainly in the nucleus. The merged images indicated that SMILE and GR, CAR, or HNF4α were colocalized to the nucleus (Figure 3H–J). Collectively, these data reveal that SMILE interacts and colocalizes with receptor GR, CAR and HNF4α *in vivo*.

### Dimerization of SMILE is not required for its repressive function

It was reported that SMILE can homodimerize through the leucine zipper region like other bZIP proteins (1,34) and this homodimerization is important for the function of leucine zipper protein (35). To determine whether the homodimerization is essential for the repressive function of SMILE, site-directed mutational analysis and *in vivo* GST pull-down assays were performed. We generated a mutant SMILE [SMILE-L (239–267) V], in which five consecutive leucine residues in the leucine zipper region were mutated to valine (Figure 4A). Next, the possibility of homodimer formation was investigated via *in vivo*

GST pull-down assays. As expected, Flag-SMILE was shown to be coprecipitated with GST-SMILE. However, Flag-SMILE-L (239–267)V was not coprecipitated with GST-SMILE-L (239–267)V (Figure 4B, upper panel), although the mutant proteins were expressed to comparable levels as the wild-type SMILE (Figure 4B, middle and lower panel). These results indicate that SMILE is capable of forming homodimers, and the mutation of the five consecutive leucine residues in the leucine zipper region destroyed the homodimerization. Next, the functional effects of the mutation were assessed using reporter assays. SMILE-L (239–267)V repressed GR- and CAR-mediated transcriptional activity as profoundly as the wild-type SMILE (Figure 4C and D). Although the repression of SMILE-L (239–267)V on HNF4α was not so strong as wild-type SMILE, SMILE-L (239–267)V still significantly inhibited HNF4α (Figure 4E). Collectively, these results indicate that SMILE homodimerization is not essential for its repressive function.

Interaction domain mapping of SMILE with GR, CAR and HNF4α

To identify the interaction domain of SMILE with the NRs, a series of SMILE deletion fragments (Figure 5A) were cloned into *in vivo* GST vector and *in vivo* GST pull-down assays were performed. We found that the mutant
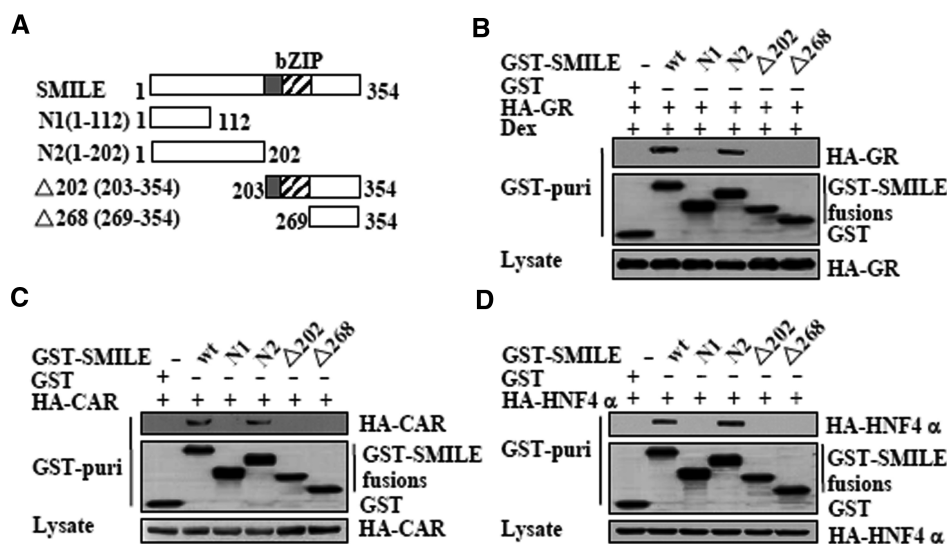
Figure 5. Interaction domain of SMILE. (**A**) Schematic representation of the structures of SMILE mutants. bZIP indicates the basic region leucine zipper domain. The numbers in the figure indicate the amino acid residues. (**B–D**) *In vivo* interaction assays between wt SMILE or SMILE mutants and GR (B), CAR (C) or HNF4α (D). 293T cells were cotransfected with expression vectors for HA-GR (B), Flag-CAR (C) or HA-HNF4α (D) with pEBG alone (GST) or pEBG-SMILE (GST-SMILE) fusions as indicated. The in vivo GST pull-down assays in (B) was performed in the presence or absence of GR ligand Dex (100 nM). The complex formation (top panel in B–D, GST puri.) and the amount of HA-GR, Flag-mCAR or HA-HNF4α used for the in vivo-binding assay (bottom panel in B–D, Lysate) were determined by western blot using an anti-HA or anti-Flag antibody. The same blot was stripped and reprobed with an anti-GST antibody (middle panel in B–D) to confirm the expression levels of the GST fusion proteins (GST-SMILE fusions) and the GST control (GST). wt, wild type. The data shown are representative of at least three independent experiments with similar results.

GST-SMILE-N2 (1–202 aa) and GST-SMILE were associated strongly with GR, CAR and HNF4α, whereas the mutants GST-SMILE-N1 (1–112 aa), GST-SMILE-Δ202 (203–354 aa) and GST-SMILE-Δ268 (269–354 aa) were not significantly associated with the NRs (upper panel in Figure 5B–D). Moreover, all the GST SMILE fusion proteins and GR, CAR, HNF4α proteins were expressed properly (middle and lower panel in Figure 5B–D), indicating that the differences in the interactions between the SMILE mutants and the NRs are not the result of differences in protein expression. Taken together, these results indicate that the region spanning residues 113–202 of SMILE is responsible for the interactions with the NRs.

To identify the region of GR, CAR and HNF4α involved in the interactions with SMILE, *in vitro* GST pull-down experiments were performed using various GR (Figure 6A), mCAR (Figure 6C) and HNF4α-deletion constructs (Figure 6E). GST-SMILE was observed to bind to ^{35}S-labeled full length GR in the presence of ligand (Figure 6B, upper panel), as well as ^{35}S-labeled GR-LBD (532–783 aa) (Figure 6C, lower panel), but did not bind to GR-N (1–531 aa) (Figure 6B, middle panel), indicating the LBD of GR is important for the ligand-dependent interaction between GR and SMILE. In addition, deletion of AF2 domain abolished the interaction of CAR with SMILE (Figure 6D) indicating that the AF2 domain of CAR is essential for the interaction. In the case of HNF4α, GST-SMILE was capable of interacting with full length HNF4α and HNF4αCD (1–370 aa) (Figure 6F, upper and middle panel), which harbor the complete LBD domain, but was not able to interact with HNF4αΔLBD

(1–174 aa) (Figure 6F, lower panel), thereby indicating that the LBD domain of HNF4α is required for the interaction between HNF4α and SMILE. Collectively, these results suggest that the LBD/AF2 domain of GR, CAR and HNF4α are essential for the interactions with SMILE.

SMILE competes with coactivators

It has been well established that a host of coactivators, including PGC-1α, CBP/p300 and GRIP1, can interact with the LBD/AF2 region of NRs to form LBD-coactivator complexes and positively regulate NR-mediated transcription (25,36,37). The aforementioned results that SMILE interacts with the LBD/AF2 region of GR, HNF4α and CAR prompted us to determine whether SMILE could compete with coactivators. In the presence of ligand, overexpression of GRIP1 increased GR-stimulated transcriptional activity (Figure 7A), which is consistent with previous report (37), and overexpression of SMILE reduced the coactivation in a dose-dependent manner (Figure 7A). In a reciprocal experiment, the transfection of increasing quantities of GRIP1 expression vector induced a gradual release of SMILE repression on GR (Figure 7A). Interestingly, SMILE overexpression also reduced PGC-1α-enhanced HNF4α-, and CAR-stimulated transactivation in a dose-dependent fashion, and overexpression of PGC-1α recovered the inhibitory effect of SMILE on HN4α and CAR (Figure 7B and C). These results indicate that SMILE can compete with the coactivators GRIP1 and PGC-1α functionally *in vivo*. To further confirm the competition between SMILE and either GRIP1 or PGC-1α, *in vitro* GST pull-down assays
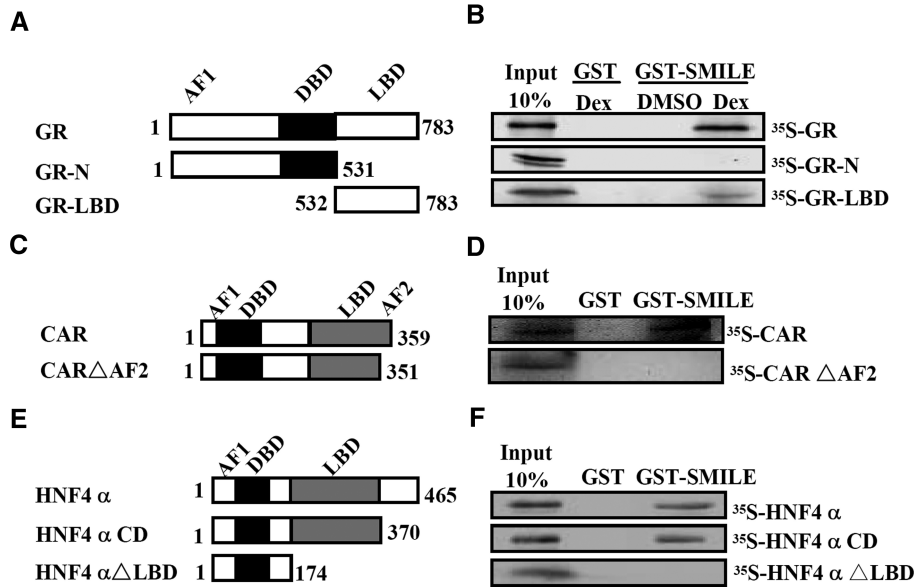
Figure 6. SMILE interacts with LBD/AF2 domain of the NRs. (**A, C** and **E**) Schematic representation of the structures of the GR (A), CAR (C) and HNF4α (E) mutants. AF1, activation function-1 domain; DBD, DNA-binding domain; LBD, ligand-binding domain; AF2, activation function-2 domain. (**B**) ^{35}S-radiolabeled GR proteins were incubated with GST, or GST-SMILE fusion proteins in the presence of ligand Dex (100 nM) or vehicle (DMSO). (**D** and **F**) ^{35}S-radiolabeled CAR (D) or HNF4α proteins (F) were incubated with GST, or GST-SMILE fusion proteins. The input lane represents 10% of the total volume of *in vitro*-translated proteins used for the binding assay. Protein interactions were detected via autoradiography. The data shown represent at least three independent experiments with similar results.
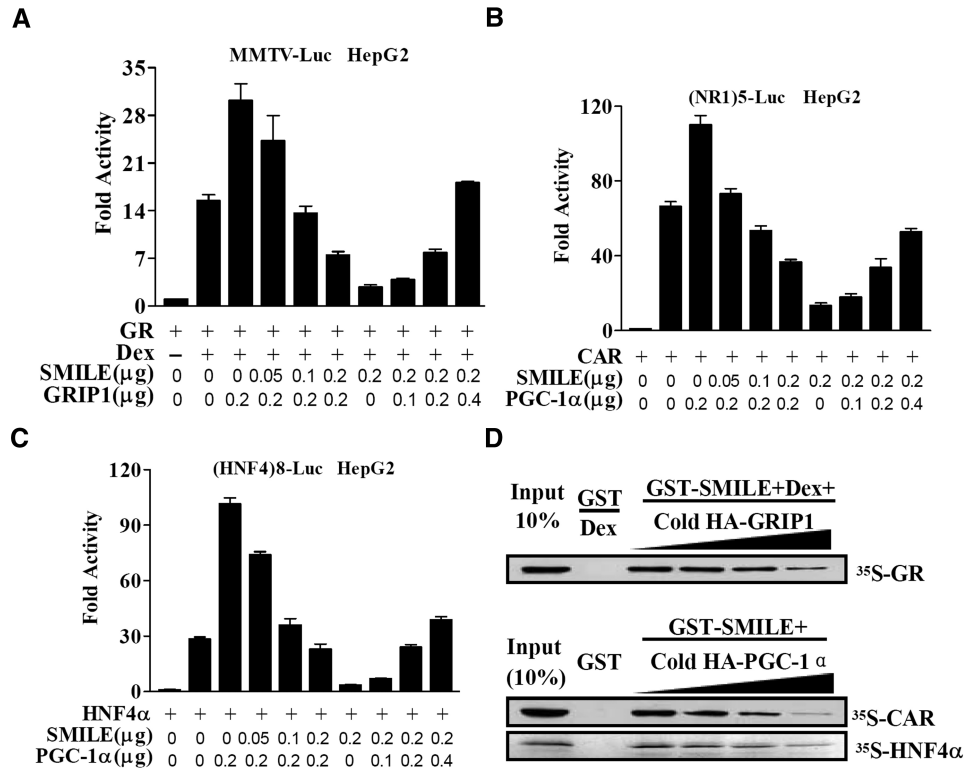
Figure 7. SMILE competes with coactivators GRIP1 and PGC-1α. Reporter assays in (**A–C**) were performed as described in the Materials and methods section. The mean and standard deviation (*n* = 3) of a representative experiment are shown. HepG2 cells were cotransfected with 0.1 µg of indicated reporter plasmids, MMTV-luc (A), (NR1)5-luc (B), or (HNF4)8-Luc (C), and 0.1 µg of pcDNA3-HA-GR (A), pcDNA3-HA-mCAR (B) or pcDNA3-HA-HNF4α (C), together with the indicated quantities of pcDNA3-Flag-SMILE, pSG5-HA-GRIP1 (A) or pcDNA3-HA-PGC-1α (B and C). Twenty-four hours after transfection, the cells were treated with or without GR ligand Dex (100 nM) for 24 h prior to the measurement of luciferase activity. (**D**) *In vitro* competition between SMILE and GRIP1 or PGC-1α. ^{35}S-radiolabeled GR (in the presence of 100 nM DEX), or CAR, or HNF4α proteins were incubated with GST, or GST-SMILE fusion proteins, together with an increasing amounts of unlabeled *in vitro* translated GRIP1 (0, 3, 6 or 12 µl, upper panel) or PGC-1α (0, 3, 6 or 12 µl, middle and lower panel) proteins. After pull-down, the beads were washed and the samples separated on a 12% SDS–PAGE gel and the protein interactions were detected via autoradiography. The data shown represent at least three independent experiments.
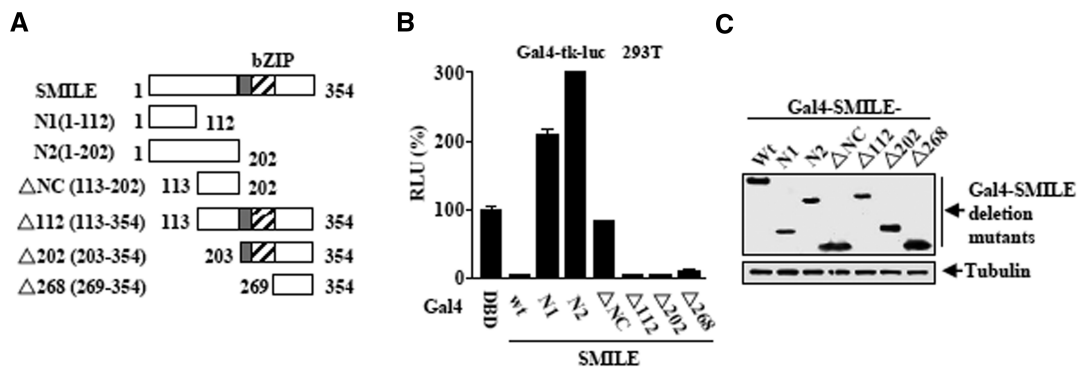
Figure 8. Intrinsic repressive function of SMILE. (**A**) Schematic representation of wt SMILE and its deletion mutants fused in-frame to the yeast Gal4 DBD-(1–147 amino acid). bZIP, basic region leucine zipper domain. (**B**) 293T cells were cotransfected with the reporter plasmid Gal4-tk-Luc and the indicated expression vectors of Gal4-SMILE or Gal4-SMILE deletion mutants together with pCMV-β-gal vector. Forty-eight hours after transfection, the luciferase activity was measured as described in the Materials and methods section. The normalized luciferase activity values are shown as the percentage of the Gal4-tk-Luc reporter activity stimulated by Gal4-DBD. The mean and standard deviation (*n* = 3) of a representative experiment are shown. (**C**) Western blot analysis of Gal4-SMILE and Gal4-SMILE deletion mutants. 293T cells were transfected with the indicated Gal-SMILE chimeras. Whole cell extracts (50 μg) were analyzed via western blot using anti-Gal4 rabbit polyclonal antibody.

were performed. In the presence of the ligand, increasing amounts of the cold competitor, HA-GRIP1, reduced the binding of ^{35}S-methionine-labeled GR protein to GST-SMILE (Figure 7D, upper panel). Moreover, increasing amounts of the cold competitor, HA-PGC-1α, reduced the association of GST-SMILE with ^{35}S-HNF4α and ^{35}S-CAR (Figure 7D, lower panel). Taken together, these results indicate that coactivator competition is one mechanism underlying for the repression of GR, HN4α and CAR by SMIILE.

SMILE has intrinsic repressive activity

Many corepressors, including SHP (38,39), and RIP140 (40), were reported to inherently possess transcriptional repressive activity. To determine whether SMILE also has an intrinsic repressive function, the transcriptional activities of a set of Gal4-SMILE deletion constructs were investigated (Figure 8A). The reporter plasmid Gal4-tk-Luc, and indicated expression vectors encoding Gal4-DBD alone, Gal4-SMILE, or Gal4-SMILE deletions were cotransfected into 293T cells. As indicated in Figure 8B, Gal4-SMILE, Gal4-SMILE-Δ112 (113–354 aa), -Δ202 (203–354 aa) showed only ∼10% of Gal4-DBD-stimulated reporter activity, and Δ268 (269–354 aa) showed only ∼15% of the activity by Gal4-DBD. However, Gal4-SMILE-N1 (1–112 aa) and Gal4-SMILE-N2 (1–202 aa) displayed 2–3-fold activity of that by Gal4-DBD, and Gal4-SMILE-ΔNC (113–202 aa) displayed a comparable effect to Gal4-DBD. Moreover, all of the Gal4-fusions were expressed properly (Figure 8C), indicating the distinct reporter activities stimulated by the Gal4-SMILE fusions were not the consequence of different protein levels. Taken together, these results indicate that the SMILE N-terminus (1–112 aa) has intrinsic activation activity, whereas the C-terminus (203–354 aa) has intrinsic repression. As a whole, SMILE showed repression activity, indicating the intrinsic repression derived from the C-terminus predominates.

SMILE recruits HDACs in a NR-specific manner

It has been reported previously that the recruitment of HDAC contributes to the intrinsic repressive function of corepressors, including RIP140 (41), and SHP (42). To determine whether SMILE could also recruit HDACs, the effect of the HDAC-specific inhibitor trichostatin A (TSA) on SMILE-mediated repression was examined. The results showed that TSA treatment partially but significantly reversed the repression of GR and HNF4α by SMILE (Figure 9A and B), whereas TSA treatment did not significantly affect the repression of CAR (Figure 9C). These results demonstrate that the recruitment of HDACs is required for the inhibition of SMILE on GR and HNF4α, but not required for the inhibition on CAR, indicating that the recruitment of HDACs by SMILE might be NR-specific.

To further determine the HDACs involved in the repression of GR and HNF4α by SMILE, the potential interactions between HDACs (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5 and HDAC6) and SMILE were investigated via *in vivo* GST pull-down assays. HDAC1 (Figure 9D), and HDAC3 (Figure 9E), as well as HDAC4 (Figure 9F) were detected in the coprecipitate only when coexpressed with the GST-SMILE but not with GST alone. The expression levels of GST, GST-SMILE, Flag-HDAC1, HA-HDAC3 and Flag-HDAC4 were confirmed via western blot analysis (middle and bottom panel in Figure 9D–F). However, Flag-HDAC2, Flag-HDAC5 and Flag-HDAC6 were not detected in the coprecipitate (data not shown). These results demonstrate that SMILE specifically interacts with HDAC1, HDAC3 and HDAC4 *in vivo*, thereby indicating that the recruitment of HDAC1, HDAC3 and HDAC4 may play a role in the SMILE-mediated repression of GR and HNF4α.

To further investigate whether HDAC1, HDAC3 and HDAC4 are also involved in the repressive effect of SMILE on GR and HNF4α, reporter assays combined with siRNA-mediated knockdown of the HDACs gene expression were performed. As shown in Figure 9G,
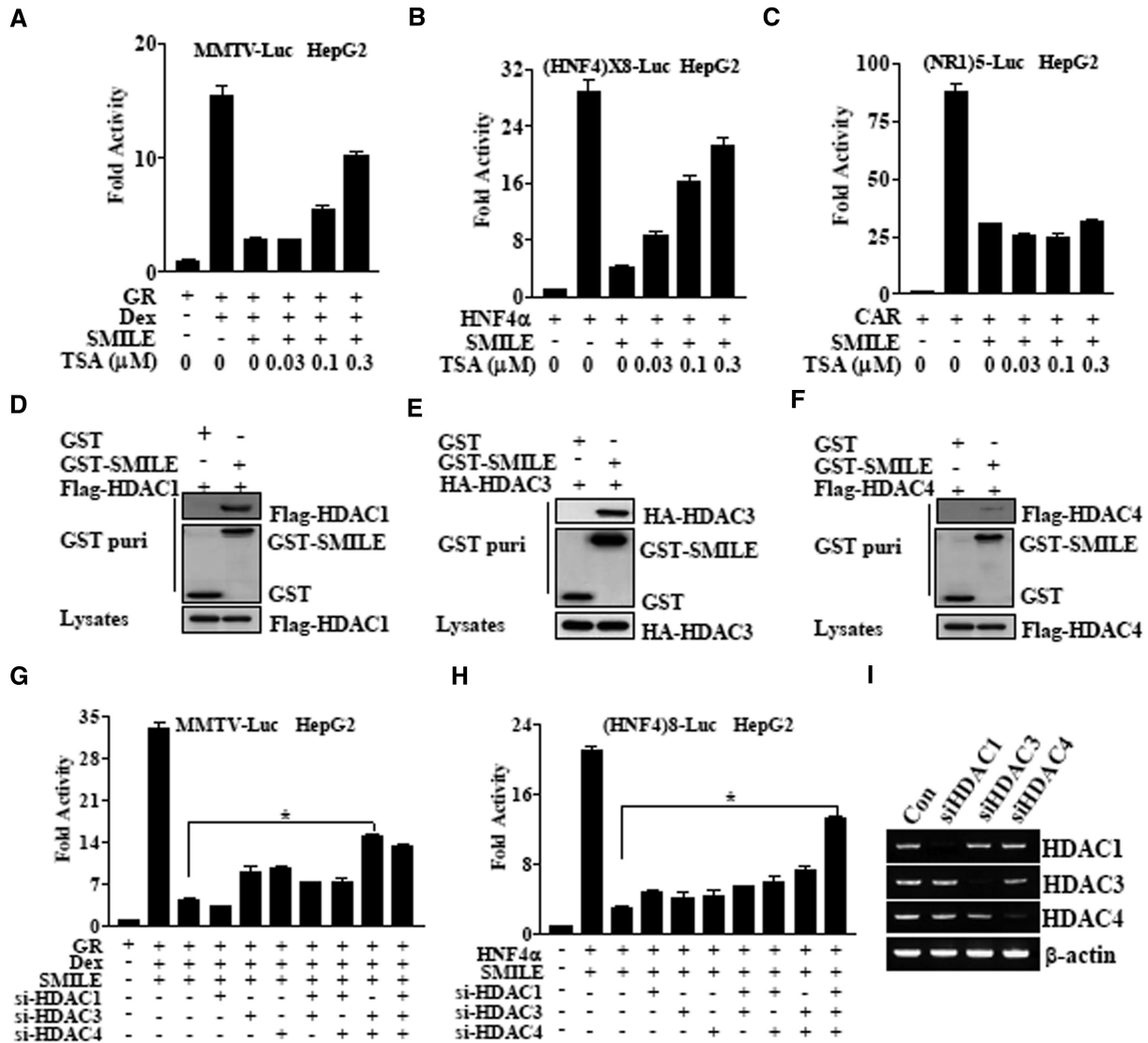
Figure 9. SMILE recruits HDACs. (A–C) TSA releases SMILE-mediated repression on GR and HNF4α. HepG2 cells were cotransfected with 0.1 μg of reporter plasmids, MMTV-luc (A), (HNF4)8-luc (B) or (NR1)5-Luc (C), and 0.1 μg of pcDNA3-HA-GR (A), pcDNA3-HA-HNF4α (B) or pcDNA3-HA-mCAR (C), together with or without 0.2 μg of pcDNA3-Flag-SMILE as indicated. Twenty-four hours after transfection, the cells were treated for 12 h with or without 100 nM of Dex. Then the cells were treated with indicated concentration of TSA for 12 h in the absence or presence of Dex (100 nM). *In vivo* interactions of HDAC1 (**D**), HDAC3 (**E**) and HDAC4 (**F**) with SMILE. 293T cells were cotransfected with expression vectors for Flag-HDAC1, or HA-HDAC3 or Flag-HDAC4 with pEBG-SMILE (GST-SMILE) or pEBG alone (GST). The complex formation (top panel in D–F, GST puri.) and the amount of Flag- or HA-tagged HDAC fusion proteins used for the *in vivo*-binding assay (bottom panel in D–F, Lysate) were determined using anti-Flag or anti-HA antibody, respecitively. The same blot was stripped and reprobed with an anti-GST antibody (middle panel in D–F) to confirm the expression levels of the GST fusion protein (GST-SMILE) and the GST control (GST). (**G** and **H**) The effect of HDAC siRNAs on the inhibition of GR- and HNF4α-mediated transactivation by SMILE. HepG2 cells were transfected with pSuper siHDAC1, siHDAC3, or siHDAC4 as indicated and after 24 h, the cells were cotransfected with 0.1 μg of indicated reporter plasmids, MMTV-luc (G), or (HNF4)8-Luc (H), and 0.1 μg of pcDNA3-HA-GR (G) or pcDNA3-HA-HNF4α (H), together with 0.1 μg of pcDNA3-Flag-SMILE and after 24 h, the cells were treated with or without 100 nM of Dex for 24 h prior to the measurement of luciferase activity. The mean and standard deviation (*n* = 3) of a representative experiment are shown. *$P < 0.05$, using Student's *t*-test. (**I**) Effect of siRNAs on the expression of HDACs. HepG2 cells were transfected with pSUPER siHDAC1, siHDAC3, siHDAC4 or pSUPER [control (con)], and after 72 h the total RNA was isolated. The mRNA levels of HDACs were measured via RT–PCR analysis, with β-actin shown as a control. The results are representative of three experiments.

siHDAC3 or siHDAC4 alone induced a slight reduction in the repression of GR by SMILE, but siHDAC1 exerted no detectable effects. Moreover, the combination of siHDAC3 and siHDAC4 additively and significantly attenuated the repression. In the case of HNF4α, only the combination of siHDAC1, siHDAC3 and siHDAC4 significantly attenuated the repression of HNF4α by SMILE

(Figure 9H). In addition, all the siRNAs for HDAC1, HDAC3, or HDAC4 were demonstrated to knockdown the specific HDAC gene expression effectively (Figure 9I). Taken together, these results indicate that HDAC3 and HDAC4 contribute to the inhibition of GR by SMILE, and HDAC1, HDAC3 and HDAC4 contribute to the repression of HNF4α by SMILE.
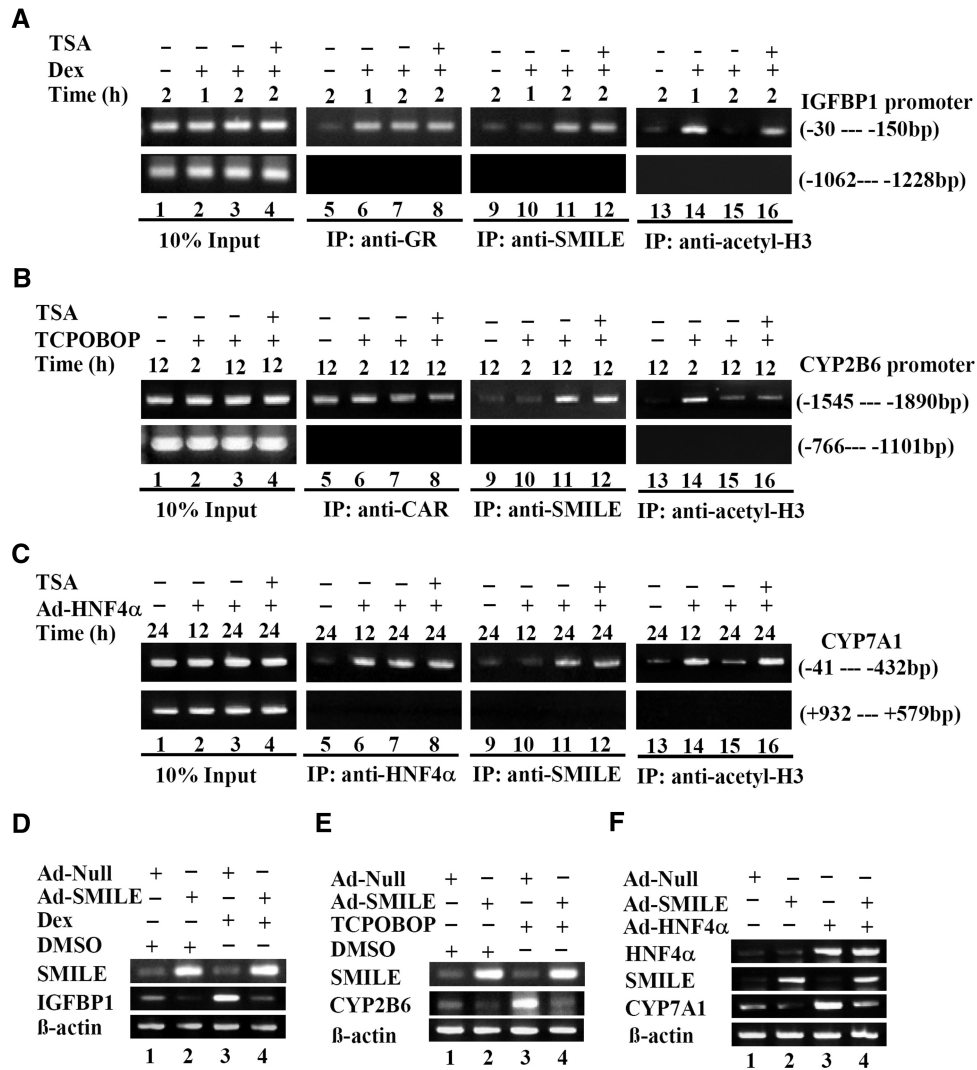
Figure 10. SMILE down-regulates the transcription of IGFBP1, CYP2B6 and CYP7A1 gene. (**A–C**) The recruitment of SMILE on IGFBP-1, CYP2B6 and CYP7A1 promoters is associated with histone deacetlyation. (A and B) HepG2 cells were stimulated with or without 100 nM of Dex (A) or 250 nM of TCPOBOP (B) in the presence or absence of 300 μM TSA as indicated. (C) HepG2 cells were infected with or without adenovirus-HNF4α (Ad-HNF4α) in the presence or absence of 300 μM TSA as indicated. Cell lysates from the treated HepG2 cells were then collected for Chip assays. Chromotian fragments were prepared and immumoprecipitated with the indicated specific antibodies. DNA fragments covering a GRE on IGFBP1 promoter (A, upper panel), or CAR-binding site on CYP2B6 promoter (B, upper panel), BARE-I and BARE-II element on CYP7A1 promoter (**C**, upper panel) were PCR-amplified as described in the Materials and methods section. The lower panels in A–C indicate the amplification of the control regions. (**D–F**) Shown are RT–PCR carried out using PCR primers for SMILE, IGFBP1, CYP2B6, CYP7A1, HNF4α and β-actin, using total RNA prepared from HepG2 cells infected with indicated adenovirus vector (Ad-null, Ad-SMILE and Ad-HNF4α) (100 pfu/cell). After 24 h of infection, the cells were stimulated with vehicle (DMSO) or 100 nM of Dex (D) for 2 h or 250 nM of TCPOBOP (E) for 24 h before total RNA was isolated. Data shown are representative of three experiments.

Adenovirus-mediated overexpression of SMILE down-regulates the expression of GR, CAR and HNF4α target genes

Next, we performed ChIP assays to determine whether SMILE can associate with the NRs on the promoter of the IGFBP1, CYP2B6 and CYP7A1 genes, which are known targets of GR, CAR and HNF4α, respectively (11,12,17). As shown in Figure 10A, low levels of GR and SMILE were associated on IGFBP1 promoter in the absence of dexamethasone (upper panel, lanes 5 and 9). We observed an increased occupancy of GR after 1 h of dexamethasone treatment (upper panel of Figure 10A,

compare lanes 5 and 6), whereas the occupancy of SMILE significantly increased after 2 h of dexamethasone treatment (upper panel of Figure 10A, compare lanes 9 and 11). On the promoter of CYP2B6, the occupancy of CAR did not significantly changed upon the treatment of CAR agonist TCPOBOP (upper panel of Figure 10B, compare lanes 6–7 to lane 5), whereas the association of SMILE was increased after 12 h TCPOBOP treatment (upper panel of Figure 10B, compare lanes 9 and 11). On the promoter of CYP7A1, the occupancy of HNF4α was significantly increased after adenovirus (Ad)-mediated overexpression of HNF4α (upper panel of Figure 10C, compare lanes 6–7 to lane 5), whereas the occupancy of

SMILE increased after 24 h of Ad-HNF4α infection (upper panel of Figure 10C, compare lanes 9 and 11). However, no recruitment was observed in the nonregulatory regions of target gene promoters (Figure 10, lower panels of A–C, see lanes 5–12). These results indicate that SMILE dynamically forms complex with GR, CAR, or HNF4α on their target gene promoters.

Since SMILE interacted with HDACs (Figure 9), we assume that the recruitment of SMILE to the target gene promoters may lead to histone deacetylation. To test this hypothesis, ChIP assays were performed using antibodies against acetylated lysine 9 of histone H3. One hour dexamethasone treatment increased the acetylation of histone H3 on the GR-binding region of IGFBP1 promoter, whereas theacetylation decreased to basal level after 2 h treatment of dexamethasone, which coincides with the timing of increased SMILE association. Interestingly, the decline of the acetylated histone H3 was recovered by HDAC inhibitor (TSA) treatment (upper panel of Figure 10A, see lanes 9–16). Moreover, 2 h TCPOBOP treatment resulted in increased acetylation of histone H3 on the CAR-binding region of CYP2B6 promoter and the acetylation diminished after 12 h TCPOBOP treatment. Although this deacetylation of histone H3 occured in line with the recruitment of SMILE, it did not change upon TSA treatment (upper panel of Figure 10B, see lanes 9–16). In addition, acetylated histone H3 on the HNF4α-binding region of CYP7A1 promoter increased 12 h after Ad-HNF4α infection and reduced to basal level 24 h after Ad-HNF4α infection, which also coincides with the recruitment of SMILE. Similar to the case of IGFBP1 promoter, the decrease in acetylated histone H3 on CYP7A1 promoter was prevented by the treatment of TSA (upper panel of Figure 10C, see lanes 9–16). Collectively, these results demonstrate that the recruitment of SMILE on these target gene promoters is associated with chromatin histone deacetylation.

As the aforementioned data show that SMILE is able to inhibit the transactivation of GR, CAR and HNF4α, and these three NRs form complex with SMILE on IGFBP1, CYP2B6 and CYP7A1 promoters, respectively, we speculated that SMILE may repress IGFBP1, CYP2B6 and CYP7A1 gene expression. As expected, the overexpression of SMILE in HepG2 cells using adenovirus vector markedly reduced dexamethasone-induced as well as the basal mRNA levels of IGFBP1 (Figure 10D, compare lane 4 to lane 3 and lane 2 to lane 1). Moreover, SMILE overexpression blocked CAR agonist TCPOBOP-mediated increase in CYP2B6 mRNA levels (Figure 10E, compare lane 4 to lane 3). In addition, SMILE overexpression also inhibited the basal and Ad-HNF4α-mediated increase in CYP7A1 mRNA levels (Figure 10F, compare lane 2 to 1 and lane 4 to 3). Taken together, these results reveal that SMILE is capable of down-regulating GR, CAR and HNF4α target gene expression.

DISCUSSION

Previous results have demonstrated that the bZIP protein SMILE plays an important role in repressing the replication of the herpes simplex virus (1,3) and serves as a coregulator in ER signaling (2). The results presented in this study extend the role of SMILE in NR signaling. SMILE inhibited GR-, HNF4α- and CAR-mediated transcriptional activity through direct binding to the LBD/AF2 domain of the NRs. Moreover, the knockdown of SMILE gene expression increased the GR, HNF4α and CAR transactivation. Furthermore, the overexpression of SMILE via adenovirus vector inhibited the transcription of the NRs' target genes, including IGFBP-1, CYP2B6 and CYP7A1. In addition, SMILE also inhibited the transactivation by receptor LXR, FXR, Nur77 and ERRγ through direct interactions (data not shown). These findings indicate that SMILE may be an important modulator of NR signaling.

We have investigated the roles of potential functional domains of SMILE for its repressive function, including the leucine zipper motif (1), the HCF-binding motif (HBM) (1,3,5) and the LXXLL motifs (NR boxes) (25,43). The leucine zipper region is known to be essential for the dimerization and functions of b-zip proteins (44). For instance, the leucine zipper of cyclic AMP response element-binding (CREB) protein is required for the dimerization and transcriptional activation (35). By way of contrast, our findings support the notion that the bZIP region of SMILE is required for the homodimerization, but is not essential for the repressive effect of SMILE on GR and CAR (Figure 4). It has been reported that Jun dimerization protein 2 (JDP-2) functions as a progesterone receptor (PR) coactivator through direct interaction via the DBD of PR and the bZIP region of JDP-2 (45). However, the domain-mapping results have demonstrated that the bZIP region of SMILE is not involved in the interactions with GR, CAR and HNF4α (Figure 5). Although HBM-mediated association of SMILE with HCF is required for SMILE to repress CREB3 (5), our reporter assay results have shown that wild-type SMILE and HBM-defective SMILE mutant (Y306A), which was demonstrated not able to interact with HCF (1), have similar inhibitory effect on GR, CAR and HNF4α (Supplementary Figure 1), indicating the repression of the NRs by SMILE is independent of HBM.

LXXLL motif is commonly found in NR coregultors and has been reported to be important for coregulators function through interaction with the LBD/AF2 domain of NRs (25,43). The results of domain-mapping analysis manifests that SMILE binds to the LBD/AF2 domain of GR, CAR and HNF4α through the region spanning residues 113–202, which contain a LXXLL motif. Surprisingly, we found that the repressive effects of SMILE on GR, CAR and HNF4α were not significantly changed by single mutation or combinatorial mutation of four LXXLL motifs (Supplementary Figure 2), indicating that LXXLL motifs are not essential for the interactions and repressive effects of SMILE in the cases of GR, CAR and HNF4α. Interestingly, this LXXLL-independent interaction was also observed between proline-rich nuclear receptor coregulatory protein (PNRC) and LBD of ERα (46). In addition of using LXXLL motifs to interact with NRs, corepressor RIP140 also uses its C-terminus, which contains no LXXLL motifs, to interact with LBD

of NRs (40). However, it remains to be determined whether the LXXLL motifs are also dispensable for the repressive effect of SMILE on other NRs, such as Nur77, LXR and FXR.

We have recently reported that SMILE functions as a coregulator in ER signaling in association with SHP. The regulation of ER by SMILE depends on the existence of SHP in breast cancer MCF-7 cells (2). In contrast, the results of our siRNA knockdown experiments indicate that SHP is not involved in the SMILE-mediated repression of GR, CAR and HNF4α (data not shown). In our previous study, SMILE regulates the inhibition of ER by SHP in a cell-type specific manner (2). However, the repression of GR, CAR and HNF4α by SMILE is not cell-type specific, since similar repressive effects were observed in 293T, HepG2 and HeLa cells (data not shown).

Our results suggest that multiple mechanisms are involved in SMILE-mediated repression. One such mechanism could be competition with coactivators such as GRIP and PGC-1α, which is a common mechanism among certain NR corepressors, including SHP (31), DAX-1 (29), RIP140 (43) and the ligand-dependent corepressor (LCoR) (47). Interestingly, besides coactivator competition, SMILE has an intrinsic repressive function, like the corepressors SHP (42) and RIP140 (41). Moreover, we found that SMILE specifically interacts with HDAC1, HDAC3 and HDAC4. The inhibition of HDAC activity using the HDAC inhibitor TSA, or the knockdown of the HDACs gene expression through siRNA partially released the repression of GR and HNF4α by SMILE. In contrast, TSA showed little effect on the repression of CAR by SMILE, indicating HDAC-dependent and -independent mechanism of repression. Consistently, our ChIP assay results also evidenced that TSA was able to prevent SMILE-associated deacetylation of histone H3 on GR and HNF4α target gene promoters, but not on CAR target gene promoter. Of note, the TSA-sensitive and -insensitive actions of SMILE are similar to several other corepessors, including RIP140 (41) and LCoR (47). In addition, HDAC1, HDAC3 and HDAC4 are required for the repression of HNF4α by SMILE, whereas HDAC1 is not essential for the repression of GR, indicating that SMILE associations with HDACs exhibits promoter specificity. Similar phenomenon has been reported with the corepressors NCoR and SMRT (26).

It is worth noting that the inhibition of DNA binding is one of the common repression mechanisms utilized by certain corepressors. For instance, this mechanism underlies the inhibition of TR and GR by tumor suppressor p53 (48,49), and the inhibition of hepatic nuclear factor-3 (HNF3) family by the corepressor SHP (30). However, our results indicate that the inhibition of DNA binding is not involved in the repression of GR, CAR, and HNF4α by SMILE, as the recruitment of SMILE exerted no detectable effect on the binding of the NRs to the promoters of IGFBP1, CYP2B6 and CYP7A1 (Figure 10A–C). Whether this mechanism is involved in the inhibitory effect of SMILE on other NRs, including Nur77, LXR and FXR, still needs to be clarified.

GR, CAR and HNF4α are crucial for liver function, including the regulation and processing of glucose, lipids, amino acids and drug metabolism, as well as bile acid homeostasis (14,15,50). Therefore, the repression of their transcriptional activity by SMILE indicates that SMILE may function as a negative coregulator in the aforementioned physiological processes. It has been reported that as integrators of various biological processes, several transcriptional coregulators are regulated by distinct nutritional and hormonal signals (51). For example, activation of cAMP signaling by fasting induces the coactivator PGC-1α expression in hepatocytes, whereas the activation of insulin-signaling pathway by refeeding exhibits quite opposite effect (51). Increased bile acid levels switch on the feedback pathway of bile acid synthesis through induction of the corepressor SHP (52). Therefore, it would be necessary to study the regulation of SMILE gene expression by diverse physiological settings and intracellular signaling pathways, which is currently under investigation. Moreover, to better understand the function of SMILE in those aforementioned physiological processes, the SMILE knockout and transgenic animal model will be useful. In addition, the identification of more SMILE-interacting proteins and the elucidation of SMILE crystal structure will be helpful to illuminate the detailed mechanism of SMILE-mediated repression.

In summary, we have identified that SMILE represses GR-, CAR- and HNF4α-mediated transactivation through direct interaction. At least two mechanisms are involved in SMILE-mediated repression of the NRs, competition with coactivators, and active repression through the recruitment of HDACs. Taken together, these observations indicate that SMILE is novel corepressor and may play an important role in NR signaling.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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