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Role of the hinge region of glucocorticoid receptor for HEXIM1-mediated transcriptional repression

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ABSTRACT

We previously reported that HEXIM1 (hexamethylene bisacetamide-inducible protein 1), which suppresses transcription elongation via sequestration of positive transcription elongation factor b (P-TEFb) using 7SK RNA as a scaffold, directly associates with glucocorticoid receptor (GR) to suppress glucocorticoid-inducible gene activation. Here, we revealed that the hinge region of GR is essential for its interaction with HEXIM1, and that oxosteroid receptors including GR show sequence homology in their hinge region and interact with HEXIM1, whereas the other members of nuclear receptors do not. We also showed that HEXIM1 suppresses GR-mediated transcription in two ways: sequestration of P-TEFb by HEXIM1 and direct interaction between GR and HEXIM1. In contrast, peroxisome proliferator-activated receptor γ-dependent gene expression is negatively modulated by HEXIM1 solely via sequestration of P-TEFb. We, therefore, conclude that HEXIM1 may act as a gene-selective transcriptional regulator via direct interaction with certain transcriptional regulators including GR and contribute to fine-tuning of, for example, glucocorticoid-mediated biological responses.

Glucocorticoid hormone is essential for maintenance of homeostasis and its actions are believed to exclusively be mediated via binding to its cognate receptor glucocorticoid receptor (GR) [1]. The GR is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily. In particular, the GR, mineralocorticoid receptor (MR), androgen receptor (AR), and progesterone receptor (PR), sharing a common structure, compose subfamily NR3C, and are also called oxosteroid receptors because their cognate ligands have C3-ketone group in the steroid A-ring [2]. GR-mediated transcriptional regulation is originally proposed to be mediated via binding of GR to the glucocorticoid response elements (GRE) on the target gene promoters, however, currently considered to be controlled by communication with a variety of cellular factors [3,4]. The GR protein consists of six distinct domains. The N-terminal A/B domains include transactivational function domain 1 (AF-1). The central C-domain constitutes the DNA-binding domain (DBD). The C-terminal E/F-domains contains the ligand-binding domain (LBD) and transactivational function domain 2 (AF-2). The D-domain, which is called hinge region as well, is relatively less conserved across nuclear receptors, and, concerning GR, was initially suggested to be a flexible linker between the DBD and the LBD, allowing proper DNA binding, dimerization, and nuclear translocation of the receptor [5]. Although several reports have raised the possibility that this hinge region mediates the interaction with a certain classes of nuclear proteins [6,7], its precise role in functional regulation of GR remains unknown.

Transcription is a complex process controlled at various steps, such as initiation, elongation, and termination [8]. Recently, it has become evident that the positive transcription elongation factor b (P-TEFb), which is composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1), stimulates transcription elongation via phosphorylating the C-terminal domain of RNA polymerase II [9]. A nuclear protein HEXIM1 (hexamethylene bisacetamide-inducible protein 1) negatively regulates this transcription elongation via sequestration of P-TEFb using 7SK small nuclear RNA as a scaffold [10]. HEXIM1 consists of an N-terminal self-inhibitory domain, a C-terminal inhibitory domain, and a central basic region (BR) conveying nuclear localization signal (NLS) and 7SK-binding domain [10]. On the other hand, a growing body of evidence indicates that HEXIM1 has other functions for modulation of gene expression. For example, HEXIM1 has been shown to directly bind and modulate the activities of transcription factors including ERα [11], GR [12], and CCAAT/enhancer-binding protein α (C/EBPα [13].

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We previously showed that GR binds to the BR of HEXIM1 to form a separate complex distinct from the HEXIM1/7SK/P-TEFb complex [12]. Moreover, overexpression of HEXIM1 decreases ligand-dependent association between GR and a coactivator transcription intermediate factor-2 (TIF2), resulting in suppression of glucocorticoid-responsive gene activation [12]. Since GR inhibition by HEXIM1 was still preserved even after antisense-mediated knockdown of 7SK, we speculated that HEXIM1 could directly suppress GR-mediated transactivation independent of inhibition of transcription elongation [12]. In the present study, we studied molecular details of this GR-HEXIM1 interaction and revealed that the hinge region of GR is responsible for the interaction with HEXIM1. Moreover, we indicated that HEXIM1 BR differentially modulates two distinct gene regulatory pathways: P-TEFb-dependent transcription elongation and suppression of GR-mediated transactivation via direct binding to the hinge region of the receptor.

Materials and methods

Reagents, antibodies, and cells. Dexamethasone (DEX) and troglitazone (TGZ) were purchased from Sigma. Other reagents were from Nacalai Tesque (Kyoto, Japan) unless otherwise specified. Anti-CDK9 antibody was obtained from Santa Cruz Biotechnology. COS-7 and HeLa cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere at 37 °C with 5% CO_2.

Recombinant DNA. cDNAs of human MR, retinoic acid receptor α (RARα), retinoid X receptor α (RXRα), AR, peroxisome proliferator-activated receptor γ (PPARγ), vitamin D receptor (VDR), and farnesoid X-activated receptor (FXR) were kindly gifted from Drs. R.M. Evans (the Salk Institute, La Jolla, CA), H. Nawata (Kyushu University, Fukuoka, Japan), E.A. Jansson (Karolinska Institutet, Stockholm, Sweden), K. Umesono (Kyoto University, Kyoto, Japan), and D.J. Mangelsdorf (University of Texas, Dallas, TX), respectively. The expression plasmids for human wild-type and various truncated GR and other nuclear receptors were generated by cloning appropriate PCR fragments into pCMX-HA vector as described before [14]. The expression plasmids for the wild-type and BR mutated FLAG-HEXIM1 and glutathione-*S*-transferase (GST)-fused HEXIM1 were generous gifts from Dr. Q. Zhou (UC Berkeley, CA) and described previously [12]. To construct expression plasmids for deletion mutant of the hinge region of GR (488–520 and 491–515a.a.) and PPARγ (173–288a.a.) (pCMX-HA-GRdD1, pCMX-HA-GRdD2, and pCMX-HA-PPARγdD, respectively), cDNAs encompassing the deleted regions with appropriate flanking sequences containing XhoI cloning site were amplified with PCR and subcloned into pCMX-HA-GR and pCMX-HA-PPARγ. To construct the chimeric protein expression plasmids pCMX-HA-GRdD1 + MRD, pCMX-HA-GRdD1 + PPARγD, and pCMX-HA-PPARγdD + GRD, we opened pCMX-HA-GRdD1 and pCMX-HA-PPARγdD at XhoI site, and the cDNA fragments encompassing the hinge region of MR (670–704a.a.), PPARγ (173–288a.a.), and GR (488–520a.a.) were subcloned, respectively. The GRE- and PPAR-response element (PPARRE)-driven reporter plasmids p2xGRE-LUC and p3xPPARRE-LUC, respectively, were described previously [12] and gifted from Dr. E.A. Jansson, respectively.

In vitro protein–protein interaction studies. To obtain the proteins of each nuclear receptor, *in vitro* transcription and translation was performed using various pCMX-HA plasmids as template with the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S] Met (1000 Ci/mmol, Amersham Biosciences). Ten microliters of each protein was added to FLAG-HEXIM1-immobilized FLAG-affinity resin or GST- or GST-HEXIM1-immobilized glutathione Sepharose beads and incubated in binding buffer (25 mM Tris–HCl, pH 7.9, 1 mM DTT, 50 mM NaCl, 1 mM PMSF, and 0.1% (v/v) NP-40) at room temperature for 90 min. Then, the beads were washed, and bound proteins were eluted with 1 M NaCl, analyzed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and followed by fluorography.

Sequence alignment. Pretty in SeqWeb package (Genetics Computer Group, Accelrys Inc.) was used with default parameters to align the hinge region sequences of GR to that of the other members of the nuclear receptors.

FLAG-affinity purification. Whole cell extracts were prepared from various FLAG-tagged HEXIM1 expressing HeLa cells, applied to Anti-FLAG M2-agarose beads (Sigma–Aldrich), and incubated for 2 h at room temperature. The beads were washed and bound proteins were eluted with SDS-sample loading buffer, and subjected to Western blot analysis using appropriate antibodies.

Transfection and reporter gene assay. Cells were cultured on 6-cm diameter culture dishes and cell culture medium was replaced with OPTI-MEM medium lacking phenol red (Invitrogen) before transfection. Total amount of the plasmids was kept constant by adding appropriate empty vectors and transfection was performed with TransIT-LT1 (Takara). After 6 h of incubation, media were replaced with fresh OPTI-MEM, and the cells were further cultured with ligands for 24 h at 37 °C. Whole cell extracts were prepared and luciferase enzyme activities were determined using Luciferase Assay System (Promega). Relative light units were normalized to the protein amounts determined with BCA Protein Assay Reagent (PIERCE).

Results

The hinge region of GR was essential for the interaction between GR and HEXIM1

To address the protein–protein interaction of GR with HEXIM1, we investigated which region of GR is essential for the interaction with HEXIM1. We synthesized various [^{35}S] Met-labeled GR mutants *in vitro* (Fig. 1), and applied them onto FLAG-affinity resin bound FLAG-tagged HEXIM1. Since, we preliminarily showed that neither AF-1 nor DBD is prerequisite for the interaction [12], we focused on the relatively C-terminal part of GR including the hinge region and the LBD/AF-2 (D, E, and F domain). As shown in Fig. 1, HEXIM1 bound to not only wild-type GR but also GR 417–777, 487–777, 417–750, 417–640, and 417–520. However, GR 1–489, 1–417, 520–777, and 417–487 did not bind to HEXIM1. These results indicated that the amino acids spanning 487–520, which overlaps with the hinge region, are important for binding to HEXIM1.

Oxosteroid receptors showed amino acid sequence homology in their hinge regions and bound to HEXIM1 in vitro

Among nuclear receptors, amino acid sequence homology of the hinge region was hardly seen except for the oxosteroid receptor subfamily including GR, MR, AR, and PR (Fig. 2A). Given this, we tested whether the representative members of the nuclear receptor superfamily interact with HEXIM1. For that purpose, GST-fused HEXIM1-immobilized beads were incubated with *in vitro* translated nuclear receptors as indicated in Fig. 2B. As expected, HEXIM1 significantly bound to not only GR but also MR and AR, but to neither PPARγ, RARα, RXRα, VDR, nor FXR (Fig. 2B). To further examine the role of the hinge region for the interaction between GR and HEXIM1, we applied domain deletion and swap analyses (Fig. 2C). Deletion of the hinge region (amino acids 488–520 and 491–515) from GR (GRdD1 and GRdD2, respectively) lost the ability of GR to bind to HEXIM1. GRdD1 + MRD, in which the GR hinge

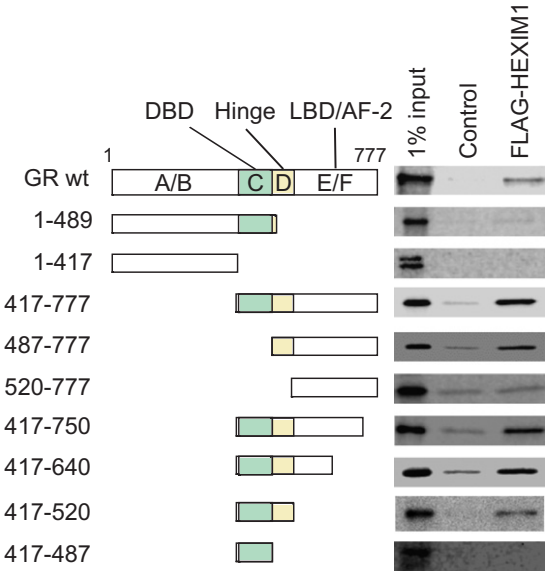

Fig. 1. HEXIM1 interacts with the hinge region of GR. Primary structures of the wild-type and mutant GR are schematically illustrated on the *left*. Numbers depict the positions of amino acids and boxes show the domain structures. FLAG-fused HEXIM1-immobilized beads were incubated with *in vitro* translated [^{35}S]Met-labeled GRs, bound proteins were analyzed on SDS–PAGE followed by fluorography, and representative results are shown.

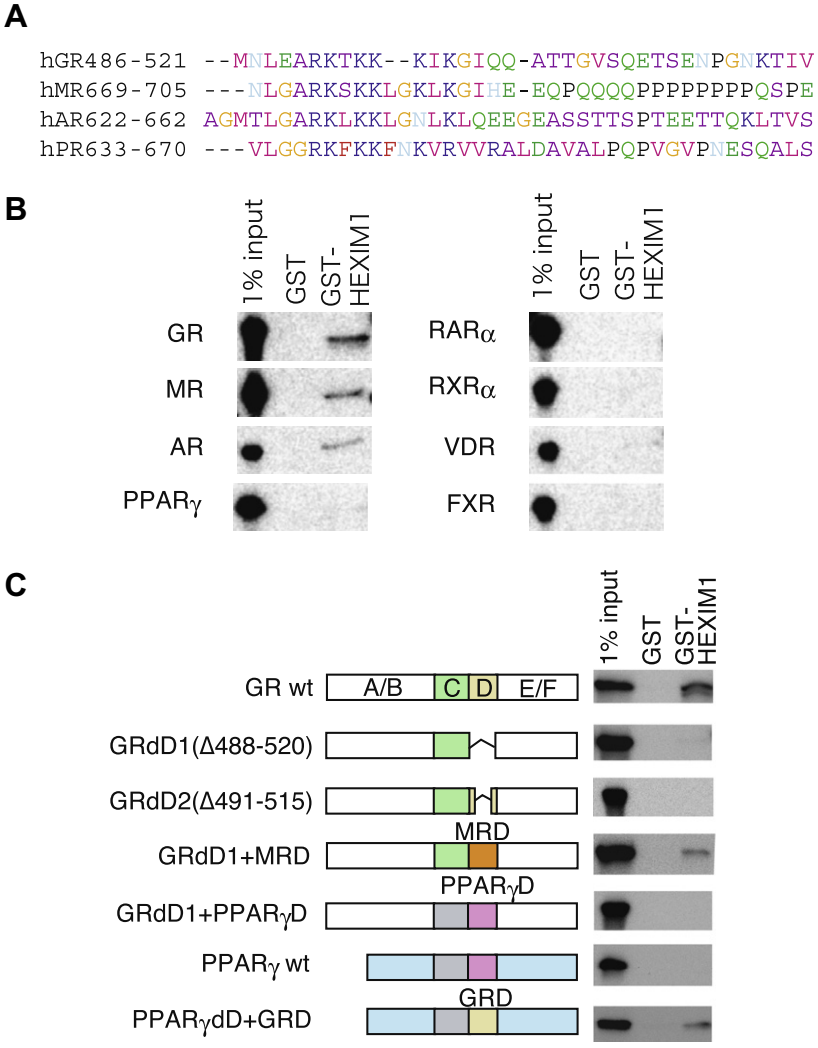

Fig. 2. The structure of the hinge region of the nuclear receptors determines their binding ability to HEXIM1. (A) Pretty in SeqWeb package (Accelrys Inc.) was used with default parameters to align the hinge sequence of GR to those of the all other members of the nuclear receptors. Homology was not significantly detected except for MR, AR, and PR and the multiple alignments of their hinge regions are shown. Dashes represent gaps in the alignment. (B,C) GST or GST-fused HEXIM1-immobilized beads were incubated with *in vitro* translated [^{35}S]Met-labeled proteins as indicated, bound receptors were analyzed on SDS–PAGE followed by fluorography, and representative results are shown. Deletion and swap mutants of the hinge region of GR, MR, and PPARγ are schematically shown on the *left* (C). D, D-domain (hinge region); MRD, PPARγD, and GRD depict the D-domain of MR, PPARγ, and GR, respectively (C).

region was substituted with that of MR, bound to HEXIM1, whereas GRdD1 + PPARγD, in which the GR hinge region was substituted with that of PPARγ, did not. Although wild-type PPARγ did not bind to HEXIM1, PPARγdD + GRD, in which the PPARγ hinge region was substituted with that of GR, acquired the ability to bind to HEXIM1 (Fig. 2C). Collectively, we concluded that HEXIM1 binds particular members of nuclear hormone receptors, especially oxosteroid receptors including GR, and that the hinge regions of those receptors may be critical for the interaction.

The role of the hinge region of GR for its transactivation function and HEXIM1-mediated suppression

Next, to analyze the role of the hinge region of GR transactivation function and HEXIM1-mediated transcriptional suppression, wild-type and various mutant GR expression plasmids (See Fig. 2) were transfected with HEXIM1 expression plasmids and GRE-luciferase reporter plasmids in COS7 cells (Fig. 3A). As previously reported, wild-type GR-activated reporter gene expression was suppressed by HEXIM1, and the hinge region-deleted mutant GRdD1 was transcriptionally silent, however, GRdD2 was capable of inducing GRE-dependent transcription albeit weakly compared with wild-type GR (refs [12,15] and Fig. 3A). Of our surprise, DEX-induced transactivation of GRdD2 was also repressed by HEXIM1 (Fig. 3A). Since not only GRdD1 but also GRdD2 lacks interaction ability with HEXIM1, these results suggest that HEXIM1-mediated GR suppression is regulated mainly by direct interaction between GR and HEXIM1, but that, in the absence of that interaction, effect of sequestration of P-TEFb by HEXIM1 becomes evident. On the other hands, GRdD1 + MRD completely preserved HEXIM1-mediated repression as well as sufficient DEX-induced transactivation, as expected from the results in Fig. 2C. In contrast, GRdD1 + PPARγD and PPARγdD + GRD did not even activate GRE- or PPARRE-driven luciferase reporter gene in the presence of DEX or TGZ, respectively (Fig. 3A and B). Together, it is indicated that, although precise mechanisms remain unknown, the integrity of the receptor architecture involving the hinge region is important in transcriptional regulation by nuclear receptor, especially oxosteroid receptors including GR. In addition, the hinge region may be essential for GR-binding-mediated repression by HEXIM1.

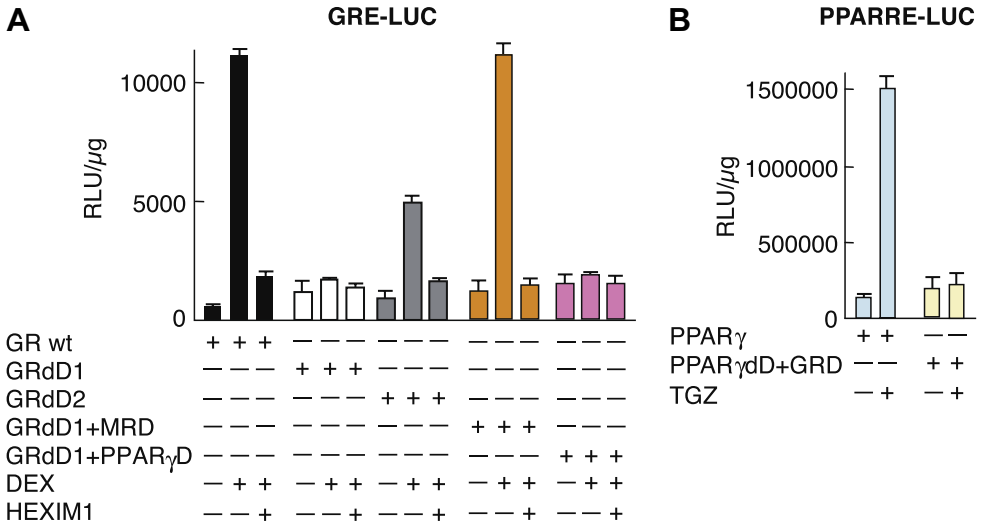

Fig. 3. The requirement of the hinge region of the nuclear receptors for their transactivation function and HEXIM1-mediated repression. (A,B) COS7 cells were cotransfected with 2 μg of reporter plasmids, pGRE-LUC (A) or pPPARRE-LUC (B), and 100 ng each of various receptor expression plasmids (also see Fig. 2C) with or without 500 ng of HEXIM1 expression plasmids as indicated, and further cultured with or without 100 nM DEX (A) or TGZ (B) for 24 h. The cell lysates were prepared for measurement of luciferase activities. All reporter gene experiments were performed in triplicate, results are expressed as relative light units (RLU) per microgram of protein in the extract, and means ± SD are shown.

HEXIM1 mutants lacking P-TEFb-binding ability suppressed GR-mediated but not PPARγ-mediated transactivation

To further analyze the molecular interplay between GR and HEXIM1, we used several HEXIM1 mutants that contain amino acid substitution in distinct portion of BR and differentially affect P-TEFb and GR activity as summarized in Fig. 4A. Those BR mutated HEXIM1 were classified according to the binding ability to P-TEFb, GR, and PPARγ, and tested in GRE- or PPARRE-driven reporter gene assays (Fig. 4B). HEXIM1 dBR + SV, which bound to neither P-TEFb, GR, nor PPARγ, did not affect reporter gene activity of PPARRE-LUC or GRE-LUC (Fig. 4B). Either HEXIM1 mutant, which could bind to GR (GR: + in Fig. 4A), suppressed ligand-dependent activation of GRE-driven reporter gene expression as well as wild-type HEXIM1, irrespective of P-TEFb-binding activity (Fig. 4B, *left panel*). In clear contrast, PPARγ-mediated activation of the reporter gene was repressed solely by wild-type HEXIM1 and 168–177A (P-TEFb: + in Fig. 4A) (Fig. 4B, *right panel*). In these experimental settings, HEXIM1 and its mutants did not affect protein expression of GR or PPARγ (data not shown). It, therefore, is indicated that direct interaction between GR and HEXIM1 plays a major role in HEXIM1-mediated suppression of GR, but that a certain category of the nuclear receptors such as PPARγ is negatively modulated by HEXIM1 solely via sequestration of P-TEFb.

Discussion

We previously revealed that HEXIM1 acts as a negative regulator of GR-mediated transcription, and that HEXIM1-binding to GR does not affect either ligand binding, nuclear translocation, or *in vitro* DNA-binding ability of GR [12]. Here we found that direct association of GR with HEXIM1 requires the hinge region of the receptor and HEXIM1 prefers oxosteroid receptors as an interacting partner among nuclear receptors. Indeed, amino acid sequence of the hinge region is least conserved across nuclear receptors, but the hinge region of GR is homologous only with those of the oxosteroid receptors (Fig. 2A). Although biological function of the very region remains largely unknown, several groups reported the biochemical interaction of the hinge region of GR with coregulatory proteins. For example, a eukaryotic cochaperone Bag1-M interacts with the hinge region of GR and negatively regulats GR action by inhibition of the ligand and DNA-binding activity of GR [6]. A nuclear receptor COUP-TFII also represses GR-induced transactivation by attracting a corepressor SMRT [7]. Moreover, it is demonstrated that the hinge region contains nuclear retention signal (NRS), overlapping with the NLS in GR [16]. In fact, the substitution of NRS dramatically weakens transactivational ability of GR [16]. We, therefore, cannot completely rule out such possibility that HEXIM1, via interacting with those coregulators or NRS, indirectly represses GR-mediated transcription. However, we previously showed that a certain fraction of HEXIM1 docks in the nucleus with GR [12], and the present study showed that the hinge region of GR is prerequisite for HEXIM1 binding at least *in vitro*. Moreover, our recent chromatin immunoprecipitation assays showed that HEXIM1 suppresses GR recruitment onto the target gene promoter (manuscript in preparation). We, therefore, favor such a model that at least part of HEXIM1 directly binds and sequesters GR to repress transcription initiation triggered by GR. Since it is generally considered that GR coactivators including TIF2 are recruited after GR binding to the promoter [4], this model may fit with our previous observation that overexpression of HEXIM1 decreases ligand-mediated GR-TIF2 association *in situ* [12]. In any case, it should be emphasized that the hinge region and its surrounding architecture may have multiple regulatory roles in GR-mediated signal transduction. The functional differences among GR, GRdD1, and GRdD2 strongly support this idea. Therefore, clarification of the molecular mechanism underlying this multimodal regulation of receptor function would be of importance to understand the integral role of the hinge region of GR.

Originally, it is estimated that majority of nuclear HEXIM1 forms complex with 7SK or Brd4, regulating expression of a large set of class II genes via intervening P-TEFb-mediated elongation reaction [10]. However, our present study rather highlights the distinct role of HEXIM1 independent from inhibition of elongation, gene-selective modulation of transcription. Recently, Montano et al. created mice carrying an insertional mutation in the HEXIM1 gene that disrupted its C-terminal region and found that the HEXIM1 C-terminal region is critical for cardiovascular development [13]. They indicated that HEXIM1 attenuates a repressive effect

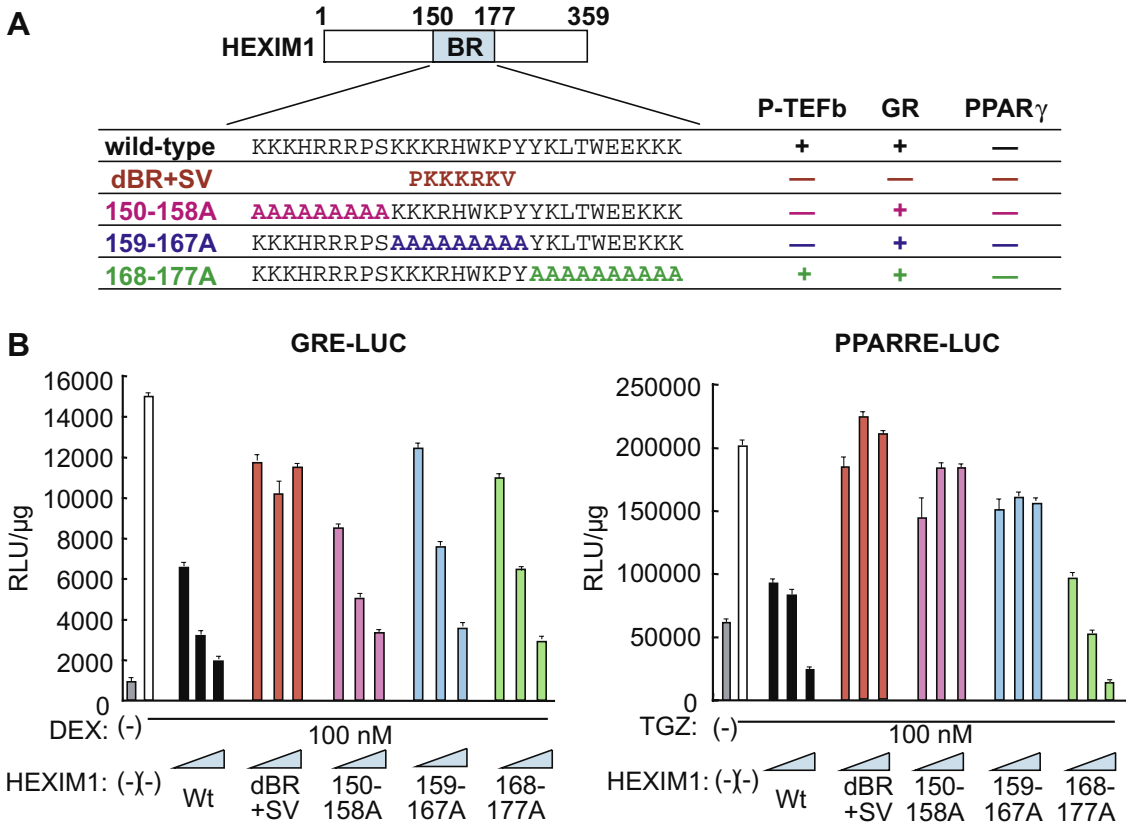

Fig. 4. HEXIM1 represses transactivation function of GR and PPARγ via functionally discrete mechanisms. (A) Primary structure of HEXIM1 (numbers depict amino acid positions), amino acid sequences of HEXIM1 mutants, and their interaction properties with P-TEFb, GR, and PPARγ are summarized. For testing P-TEFb binding, whole cell extracts of FLAG-tagged HEXIM1 or its mutants expressing HeLa cells were prepared and immunoprecipitated with anti-FLAG affinity resin, and CDK9 subunit of P-TEFb was detected using immunoblot. For testing GR and PPARγ binding to HEXIM1 and its BR mutants, various GST-fused HEXIM1-immobilized beads were incubated with *in vitro* translated [^{35}S]Met-labeled proteins, and bound receptors were analyzed on SDS–PAGE followed by fluorography. (B) COS7 cells were cotransfected with 2 μg each of pGRE-LUC (*left*) or pPPARRE-LUC (*right*) and 100 ng of expression plasmids for wild-type GR (*left*) or PPARγ (*right*), respectively, with or without increasing amounts (100, 500, and 1000 ng) of expression plasmids for HEXIM1 or its mutants as indicated. The cells were further cultured with or without 100 nM DEX (*left*) or TGZ (*right*) for 24 h and the cell lysates were prepared for measurement of luciferase activities. Experiments were performed in triplicate, results are expressed as relative light units (RLU) per microgram of protein in the extract, and means ± SD are shown.

of C/EBPα on vascular endothelial growth factor gene transcription. Note that C-terminal deletion of HEXIM1 does not affect P-TEFb inhibition [13]. Together with our study, it is strongly suggested that HEXIM1 may elicit modulation of gene expression not only via P-TEFb suppression but also via gene context-dependent mechanisms.

At this moment, many questions remain to be answered. It was reported that HEXIM1 represses transactivation function of ERα [11] which belongs to NR3A subfamily of the nuclear receptor superfamily [2]. They showed that HEXIM1 binds with ERα not via the hinge region but via the LBD [11]. Although the requirement of distinct domains for interaction with HEXIM1 between GR and ERα is interesting, we do not know how HEXIM1 can distinguish these two receptors classified in distinct subcategories of the nuclear receptor. This is also the case among oxosteroid receptors, since several members of this receptor group often are simultaneously expressed with fulfilling their own roles in the same tissue [17,18]. Similarly, it is also of particular importance to clarify how HEXIM1, using the central BR, differentially controls P-TEFb inhibition and GR suppression. Further studies, therefore, are clearly needed to address these issues. However, control of GR function *in situ* still remains to be a critical issue in medical fields [19]. Since HEXIM1 has a unique mode of modulation of GR function, HEXIM1 might be a pharmacological target for drug development to modulate GR function.

Acknowledgments

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