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Polyamine-modulated factor 1 represses glucocorticoid receptor activity

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Abstract

Polyamine-modulated factor 1 (PMF-1) has been reported to interact with NF-E2 related factor 2 (Nrf-2) and activate the polyamine-induced transcription of spermidine/spermine N^1-acetyltransferase (SSAT) gene. Polyamines are important regulators of cell growth and cell death and have been implicated in glucocorticoid-induced apoptosis. In the present study, we have identified and characterized new functional binding partners for PMF-1. Our results demonstrate that PMF-1 binds to the glucocorticoid receptor (GR). PMF-1 also represses glucocorticoid-induced transcription. Furthermore, we show that PMF-1 has an intrinsic repression activity, which could contribute to the repressive effect on GR. PMF-1 can also interact with the GR corepressor, receptor-interacting protein 140 (RIP140), but does not further enhance the repressive effect of RIP140. Our results suggest that PMF-1 has a broader function in regulation of genes and can contribute to glucocorticoid signaling.

Keywords: Glucocorticoid; Glucocorticoid receptor; Nrf-2; PMF-1; RIP140; Spermidine/spermine N^1-acetyltransferase

Polyamines e.g. putrescine, spermidine, and spermine, are polycationic compounds in living cells and are essential for cell growth (for a review, see [1]). In the cell, positively charged polyamines are metabolized via a highly regulated pathway and interact electrostatically with negatively charged DNA, RNA, and proteins. Polyamine-modulated factor 1 (PMF-1) is a cofactor for regulation of the expression of the rate-limiting enzyme in the catabolic pathway of polyamine metabolism, spermidine/spermine N^1-acetyltransferase (SSAT) [2–4]. PMF-1 interacts with the transcription factor NF-E2 related factor 2 (Nrf-2), which binds to the polyamine responsive element (PRE) in the SSAT gene promoter. PMF-1 is expressed in many human tissues like the heart, skeletal muscles, kidney, and liver. The mouse homologue of human PMF-1 shares almost 80% homology and encodes for two alternatively spliced mRNAs, PMF-1 long (PMF-1L) and PMF-1 short (PMF-1S) [4]. PMF-1 expression has been shown to be regulated by polyamines [2,4,5]. Existence of potential functional PREs in other genes besides SSAT and other PMF-1 interacting proteins are currently being elucidated. PMF-1 has been shown to interact with the human homologue to subunit 7 of the *Arabidopsis* COP9 signalosome, CSN7, and compete with it for binding to Nrf-2 [6]. COP9 signalosome proteins are playing a role in intracellular signaling and transcriptional control, suggesting that PMF-1/CSN7 interaction might have an effect in PRE-regulated SSAT gene transcription. Work on the polyamine regulated eukaryotic initiation factor 4E binding protein 1 (4E-BP1) gene identified a PRE at the 5′ flanking region that bound the Nrf-2/PMF-1 complex [5]. Since 4E-BP1 is important for protein translation it implies a role for PMF-1 in regulation of protein translation for cellular growth and differentiation. Other studies have suggested PMF-1 to be a member of a kinetochore associated multiprotein complex, involved in mediating chromosomal segregation during mitosis [7]. Thus, some physiological aspects and mechanisms of PMF-1 have been presented, but still much remains unclear.

Nuclear hormone receptors are ligand-regulated transcription factors that either activate or repress transcription

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of genes to control many aspects of physiology and metabolism. One such member is the glucocorticoid receptor (GR), which is activated by the glucocorticoid hormone and has implications in carbohydrate and lipid metabolism as well as stress and inflammatory responses (for review, see [8]). Unliganded GR is localized in the cytoplasm and held inactive by the binding of chaperone proteins such as heat shock protein 90. Upon ligand binding the chaperone proteins are dissociated and GR enters the nucleus and regulates gene transcription. Transcriptional activation by GR is mediated through the recruitment of numerous coregulators that modify the chromatin template and recruit the basal transcriptional machinery.

Receptor-interacting protein 140 (RIP140) is a unique coregulator of nuclear receptors (for reviews, see [9,10]). It mainly acts as a corepressor reducing gene transcription but, in contrast to other corepressors that interact with unliganded or antagonist-activated receptors, RIP140 interacts with ligand-activated receptors. Genetic knockout studies with mice showed that RIP140 has important physiological functions in female fertility and energy and glucose metabolism. RIP140 has been suggested to repress receptor activity by competing with the binding of coactivators to the receptor. However, RIP140 also has an intrinsic repression activity mediated through interaction with HDAC proteins and the corepressor CtBP. We have previously shown that GR interacts with RIP140 and that RIP140 acts as a corepressor on GR-regulated genes [11].

Depletion of polyamines results in cell cycle arrest, alteration in expression of many growth related genes, including cyclin-dependent kinase inhibitors $p21^{WAF-1/CIP1}$ and $p27^{KIP}$, stabilization of p53 protein and mRNA levels and changes in cyclin levels, confirming polyamines role in regulation of cell growth [12–15]. The role of polyamines as cell growth modulators has targeted their tightly regulated metabolism pathway for antineoplastic intervention, through among other synthesis of polyamine analogues that alter polyamine metabolism by e.g. inducing SSAT expression [16]. Glucocorticoids (GC) induce an irreversible G_1 cell cycle arrest, which precedes apoptosis in lymphoid cells and therefore GC are used as therapeutic agents in lymphoid cell malignancies [17]. GC are a part of the death-signaling pathway in hematology cells, reducing mitochondrial membrane potential, upregulating of Bcl-2 family genes, activating caspase complex proteins, inducing IκB and down regulating c-myc.

Interestingly, the activity of SSAT is induced in GC-induced apoptosis [18,19]. Furthermore, polyamine depletion enhances GC-induced apoptosis and addition of the polyamine spermine reduces apoptosis [20–22]. These findings suggest that polyamine levels and metabolism are involved in GC signaling.

The aim of this study was to investigate the possible role of the polyamine regulated PMF-1 in GC-induced GR activity. We show that PMF-1 interacts with GR and functionally represses GR transcriptional activation. We also find that PMF-1 interacts with the GR corepressor RIP140 but has no effect on its repressive action on GR activity. Additionally, we found that PMF-1 has an intrinsic repression activity that may partially explain PMF-1 repressive action on GR.

Materials and methods

Plasmids. Expression plasmid pCMV4-hGR, pSG5-HA-RIP140, and pM1-RIP140 and reporter plasmids p19-tk-luc, pGAL4-luc, and pCMV-βgal were described previously [23,24]. Mouse PMF-1L/pCDNA3.1 (+) was a kind gift from Robert A. Casero, Johns Hopkins School of Medicine, Baltimore, USA. The cDNA encoding mPMF-1L were amplified by PCR using the plasmid PMF-1L/pCDNA3.1 (+) as template which includes a BamHI site in the 5′ site and XhoI site in the 3′ site, respectively. Glutathione *S*-transferase (GST) fusion proteins were expressed from pGEX plasmids (Amersham Pharmacia Biotech). Plasmids expressing GST-mPMF-1L was constructed by subcloning of a BamHI–XhoI fragment from the PCR products into pGEX-4T-1 digested by both BamHI and XhoI. Plasmid expressing GAL4 DNA-binding domain (DBD)-fused mPMF-1L was made by subcloning of a BamHI–XhoI fragment from the pGEX-mPMF-1L plasmids into pM2 plasmid (Clontech) digested by BamHI and SalI. The yeast expression plasmids pGBT9-RIP140 (1–472) and pGTB9-RIP140 (431–1158) were described previously [25]. pGAD-PMF-1L was made by subcloning the fragment encoding PMF-1L into pGAD plasmid (Clontech).

Reporter gene assays. COS-7 cells were plated in 24-well plates and on the following day transfected with plasmids by using the Fugene 6 transfection reagent (Roche). The plasmid amounts used were as follows: 100 ng of p19-tk-luc, 100 ng of pGAL4-luc, 5 ng of pCMV-βgal, 2.5 ng of pCMV4-hGR, 100–200 ng of pFLAG-CMV-PMF-1L, 10 ng of pSG5-HA-RIP140, and 100 ng of pM1-RIP140. The plasmid amount was kept constant in each transfection by addition of the corresponding empty plasmid. On the next day, fresh medium containing 1 μM Dex or ethanol, as a vehicle, was added and after 24 h the cells were harvested. Luciferase and β-galactosidase assays were performed with Gen-Glow-1000 kit and Galacto-Light plus kit (Tropix), respectively, in an Anthos Lucy 3 luminometer (Anthos Labtec-instruments). All experiments were carried out in triplicate, repeated at least three times, and luciferase activity was normalized to β-galactosidase activity.

GST pull-down assays. The GST-PMF-1L was expressed in *Escherichia coli*, and extracts were prepared as previously described [26]. [^{35}S]Methionine-labeled proteins were synthesized *in vitro* with the TnT-coupled reticulocyte lysate system (Promega). GST fusion protein (10 μg) bound to 60 μL of glutathione beads (Sigma) was incubated with 17 μl of *in vitro* translated protein in 200 μl of pull-down buffer (20 mM Hepes–KOH [pH 7.9], 10% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mg of bovine serum albumin per ml, and 0.01% Igepal CA-630) overnight at 4 °C. The beads were recovered by centrifugation and washed six times with pull-down buffer without bovine serum albumin, and bound proteins were eluted with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, analyzed by SDS–PAGE, and visualized by autoradiography.

Yeast two-hybrid assay. Yeast strain AH109 (MATα, his3, ade2, mel1, and lacZ) was used in a GAL4 based yeast two-hybrid interaction assay, MATCHMAKER two-hybrid system, according to the manufacturers (Clontech). AH109 yeast cells were transformed with plasmids pGTB9-RIP140 expressing GAL4-fused RIP140 domains and pGAD expressing GAL4 activation domain fused to PMF-1. Yeast two-hybrid assays were performed in liquid culture and measured as β-galactosidase activity.

Coimmunoprecipitation. COS-7 cells were plated onto six-well plates and transfected the next day with expression plasmids using Fugene 6 transfection reagent (Roche). One microgram pSG5 empty vector or vector expressing HA-RIP140 was cotransfected with FLAG-fused PMF-1L or empty plasmid. After overnight transfection, cells were harvested in PBS, lysed in TNE lysis buffer (0.1% Igepal CA-630, 150 mM NaCl,

10 mM Tris–HCl, pH 7.8, and 1 mM dithiothreitol), and then centrifuged at 14,000*g*, 5 s, room temperature. The supernatant was incubated in normal rat immunoglobulin G-Sepharose for 30 min at 4 °C under rotation, then with anti-HA affinity matrix rat monoclonal antibody (Roche) overnight 4 °C under rotation. The matrix pellet was washed in TNE lysis buffer and bound proteins were eluted by SDS–PAGE sample buffer and analyzed by SDS–PAGE and Western blotting with anti-FLAG M5 monoclonal antibody (Sigma) and anti-HA.11 monoclonal antibody (Covance).

Results

Interaction of PMF-1 with GR

The mouse homologue of PMF-1 shares 79% homology with the human PMF-1 (Fig. 1A). Mouse PMF-1 comes in two splice variants that give form to PMF-1L and PMF-1S, which lacks 69 amino acids from the central domain of PMF-1L. It has previously been shown that PMF-1 contain coiled coil domains, that are in other proteins needed for DNA binding and gene regulation [27]. PMF-1 also contains a SUMOylation site that is found in many proteins to be required for protein repression and a corepressor nuclear receptor (CoRNR) box motif that is reported from previous studies to be important for binding of corepressors to nuclear receptors [28,29].

To determine whether PMF-1 can interact with GR *in vitro,* GST pull-down experiments with GST-fused PMF-1L and *in vitro* translated GR were performed (Fig. 1B). Interestingly, results showed that the *in vitro* translated GR interacted with PMF-1L. Together this implies that mouse and human PMF-1 have similar motifs, which may contribute to their interaction with GR and suggest a role for PMF-1 as a repressor.

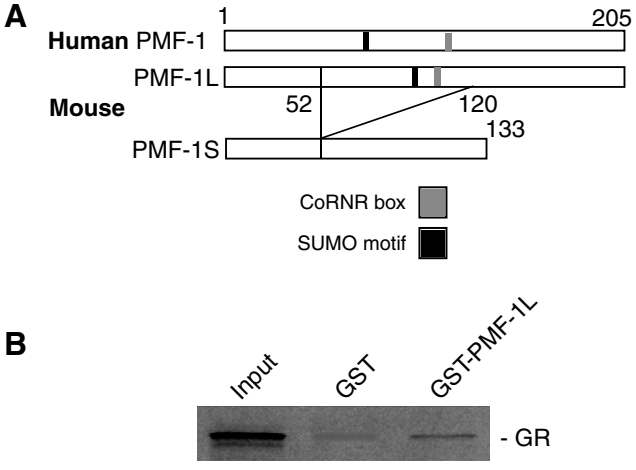

Fig. 1. PMF-1L interacts with GR. (A) A schematic representation of PMF-1L (long) and PMF-1S (short). The corepressor nuclear receptor (CoRNR) box motif and SUMOylation (SUMO) site are depicted. (B) GST pull-down assay. *In vitro* translated GR were incubated with GST or GST-PMF-1L bound to glutathione beads. The input represents 10% of the amount of labeled protein used. Input and interacting protein were visualized by SDS–PAGE and autoradiography.

PMF-1L functionally represses GR activity

To elucidate the functional role of PMF-1 on GR activity, COS-7 cells were transfected with GRE-regulated reporter gene, GR and increasing amounts of PMF-1L expressing plasmid. Ligand-induced GR showed a strong reporter gene activation in absence of PMF-1L. However at increasing levels of PMF-1L protein, PMF-1L repressed the ligand-induced GR activity (Fig. 2A).

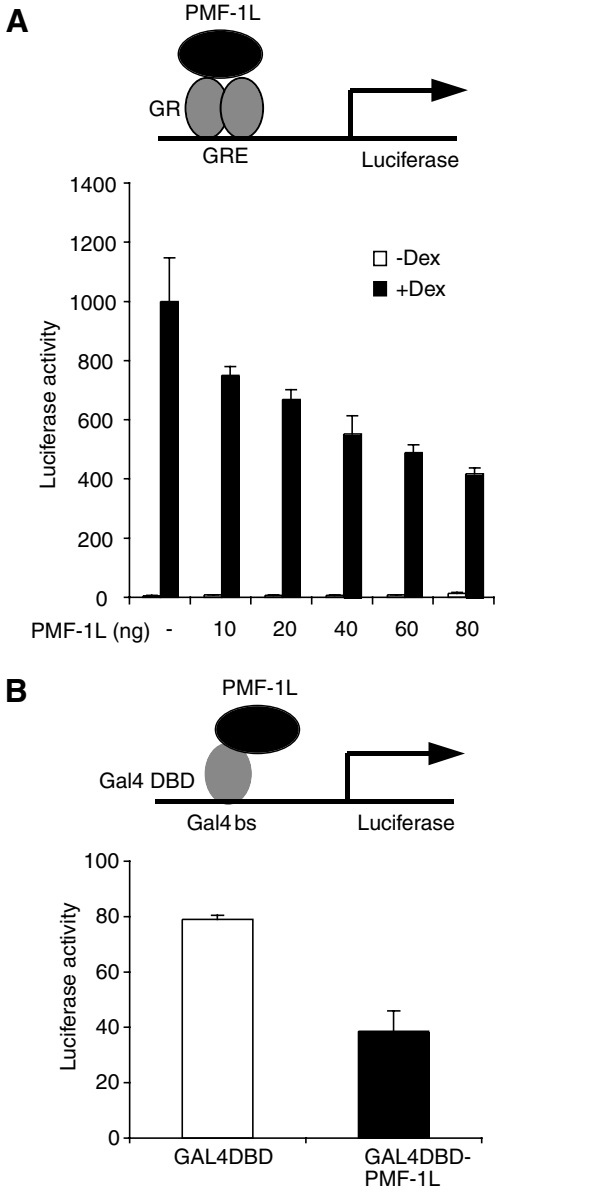

Fig. 2. PMF-1L functionally represses GR gene transcription. (A) COS-7 cells were transfected with plasmids expressing GR, reporter plasmid p19tk-luc, containing a GRE-regulated luciferase reporter gene and increasing amounts of a plasmid expressing PMF-1L. The cells were maintained in the presence of absence of 1 μM Dex. (B) COS-7 cells were transfected with plasmids expressing GAL4 DBD or GAL4 DBD-fused PMF-1L with reporter plasmid GAL4-luc, containing a luciferase reporter gene regulated by GAL4-binding sites (Gal4 bs). The mean and standard deviation ($n = 3$) of a representative experiment are shown.

Nuclear receptor corepressors like RIP140 have an intrinsic repression activity that can analyzed by fusing the repressor to GAL4 DBD and studying the induced repression of a reporter gene regulated by GAL4-binding sites. To determine whether PMF-1 has an intrinsic repression activity, COS-7 cells were transfected with GAL4 DBD-fused PMF-1L and a reporter gene regulated by GAL4-binding sites. Results showed a prominent reporter gene activity observed in absence of GAL4 DBD-fused PMF-1L protein. However, when cells were transfected with GAL4 DBD-fused PMF-1L, a PMF-1L induced repression of reporter gene activity was obtained, indicating a significant PMF-1L intrinsic repression activity (Fig. 2B).

PMF-1L interacts with corepressor RIP140

Interestingly, a yeast two-hybrid assay showed that PMF-1L also could interact with the GR corepressor RIP140 (data not shown). To validate the interaction, a GST pull-down assay was performed, where GST-fused PMF-1L and *in vitro* translated RIP140 were used. The results showed that PMF-1L interacts with RIP140 *in vitro* (Fig. 3A).

To further study the interaction in cells, we performed a coimmunoprecipitation experiment with COS-7 cells transfected with HA-fused RIP140 and FLAG-tagged PMF-1L. Precipitation of proteins from cells extracts with an anti-HA antibody coupled to agarose beads showed that PMF-1L coprecipitated with RIP140 (Fig. 3B, lane 4), but was not precipitated with an empty vector (Fig. 3B, lane 3), conforming the specific interaction of the coimmunoprecipitation.

To map the interaction domain on RIP140, the yeast two-hybrid liquid β-galactocidase assay was used. Fig. 3C shows that PMF-1L can interact both with the N-terminal (1–472) and C-terminal domain (431–1158) of RIP140.

PMF-1L does not change RIP140 repressive action on GR activity

To elucidate the possible effect of PMF-1L on the repressive action of RIP140 on GR activity, we used the GRE-regulated reporter gene assay. As previously reported RIP140 significantly repressed ligand-induced GR activity in the absence of PMF-1L (Fig. 4A). Similar to the results in Fig. 2A, PMF-1L repressed ligand-induced GR activity in the absence of RIP140 (Fig. 4A, white bars). However, RIP140 repressed GR activity to the same level in the presence and absence of PMF-1L (Fig. 4B, black bars). These results suggest that PMF-1L does not modify the RIP140 corepression of GR activity.

To determine whether PMF-1L can effect RIP140 intrinsic repression, COS-7 cells were transiently transfected with GAL4 DBD-fused RIP140, GAL4 DNA-binding reporter gene and FLAG-fused PMF-1L. The results showed that the intrinsic repression activity of RIP140 was not altered

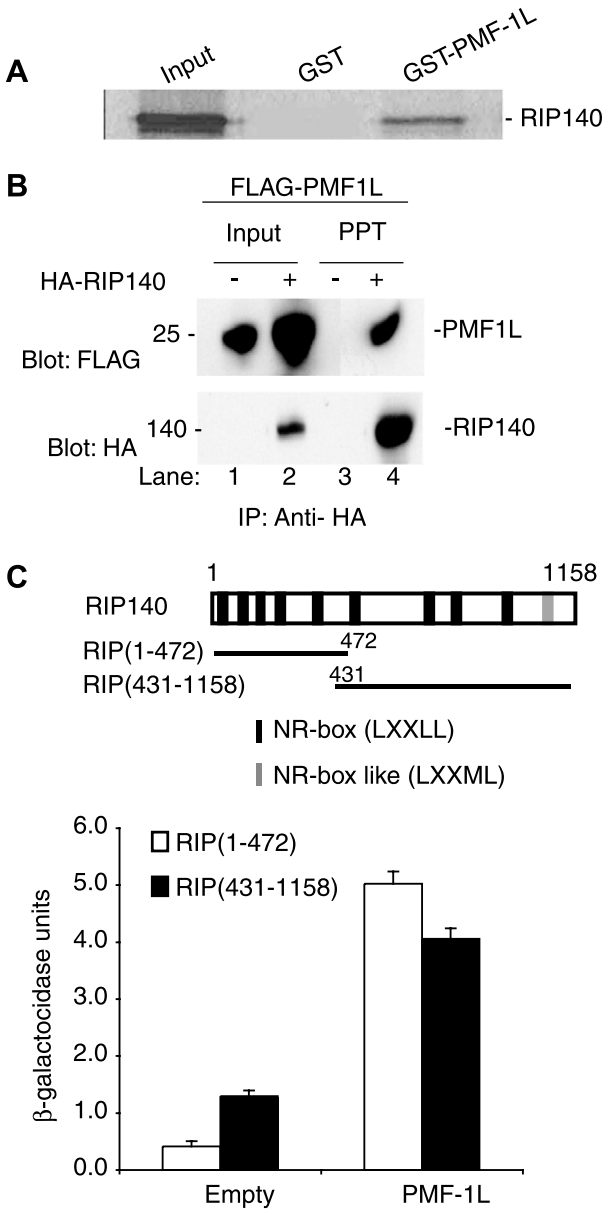

Fig. 3. PMF-1L interacts with RIP140. (A) GST pull-down assay. *In vitro* translated RIP140 were incubated with GST or GST-PMF-1L bound to glutathione beads. The input represents 10% of the amount of labeled protein used. Input and interacting protein was visualized by SDS–PAGE and autoradiography. (B) Coimmunoprecipitation assay. COS-7 cells were cotransfected with FLAG-tagged PMF-1L and either HA-tagged RIP140 (lanes 2 and 4) or empty vector (lanes 1 and 3). FLAG-PMF-1L was coimmunoprecipitated with HA-RIP140 using anti-HA antibody. The proteins in the cell extracts (input) and the anti-HA precipitated proteins (PPT) were analyzed with Western blotting using anti-HA and anti-FLAG antibodies. (C) Liquid β-galactosidase assay. Schematic representations of full-length RIP140 (RIP140), its N-terminal domain (RIP (1–472)) and C-terminal domain (RIP (431–1158)). Black and grey boxes depict the distribution of LxxLL- and LxxLL-like motifs involved in nuclear receptor binding, respectively. Results of pairwise two-hybrid analyses are shown as relative β-galactosidase units. Yeast Y187 cells harboring the *lacZ* reporter construct were co-transformed with RIP140 N-terminal (1–472) or C-terminal (431–1158) fused to the GAL4 DBD together with full-length PMF-1L fused to GAL4 activation domain or GAL4 activation domain only (empty). Cells were collected and used for β-galactosidase liquid culture assay. The mean and standard deviation ($n = 3$) of a representative experiment are shown.

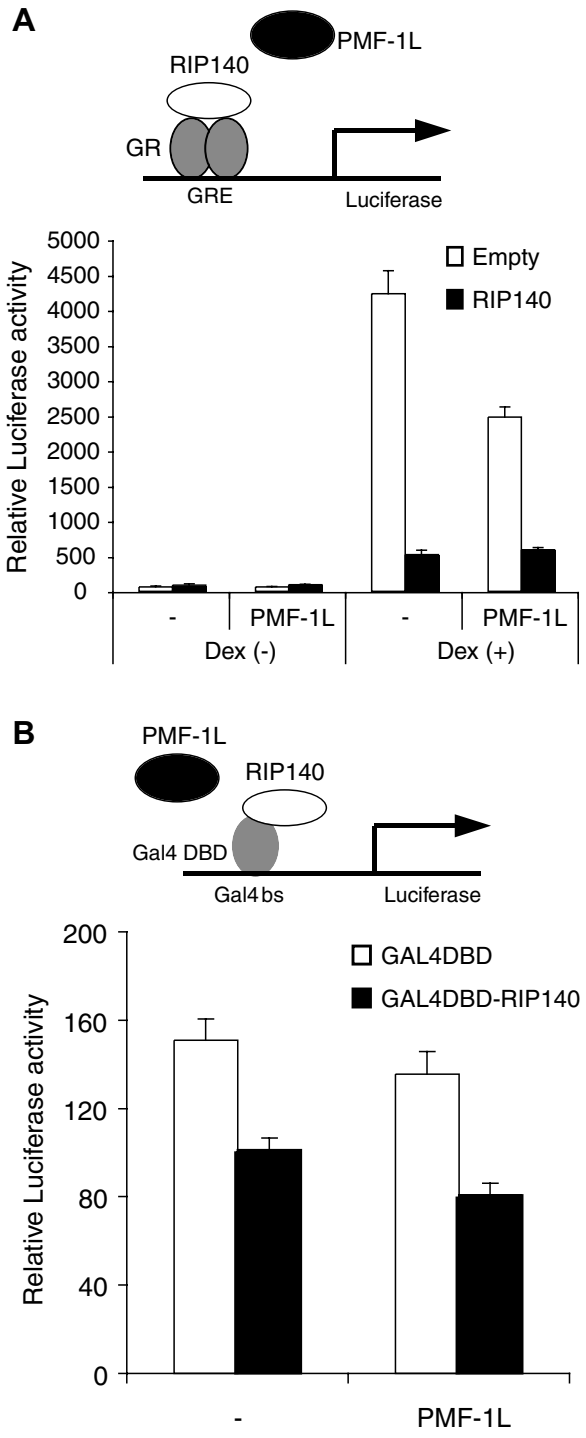

Fig. 4. PMF-1L does not change RIP140 repressive action on GR activity. (A) COS-7 cells were transfected with plasmids expressing GR, reporter plasmid p19tk-luc, containing a GRE-regulated luciferase reporter gene, FLAG-fused PMF-1L, and HA-tagged RIP140 or corresponding empty vectors. The cells were maintained in the presence of absence of 1 μM Dex. (B) COS-7 cells were transfected with plasmids expressing GAL4 DBD or GAL4 DBD-fused RIP140 with reporter plasmid GAL4-luc, containing a luciferase reporter gene regulated by GAL4-binding sites (Gal4 bs) and in presence or absence of FLAG-fused PMF-1L. The mean and standard deviation ($n = 3$) of a representative experiment are shown.

by the presence of PMF-1L (Fig. 4B). In summary, these results suggest that although PMF-1L can interact with RIP140 it does not change the repression activity of RIP140 on GR-regulated transcription.

Discussion

PMF-1 was originally identified as a polyamine induced protein that interacts with the transcription factor Nrf-2 to regulate SSAT, the rate-limiting enzyme in the catabolic pathway of polyamine metabolism [2–4]. Our results showing that PMF-1 can also interact with GR, another transcription factor, and RIP140, a transcriptional coregulator, imply that PMF-1 can have a broader function in regulation of genes. Our results show that PMF-1 represses GR-regulated transcription and has also an intrinsic repression activity. PMF-1 was previously shown to slightly enhance Nrf-2-mediated transcription [2]. Thus, it is possible that the effect of PMF-1 on gene transcription is dependent on the other transcription factors involved. Although we could demonstrate an interaction between PMF-1 and the corepressor RIP140, in our assays we could not see an effect of PMF-1 on the repression activity of RIP140, suggesting that PMF-1 cannot further enhance the repressive function RIP140. However, it is possibly that PMF-1 can in some context modulate the function of RIP140.

It is intriguing that GR can been connected to regulation of SSAT activity as suggested by the observation that SSAT is upregulated in GC-induced apoptosis [18,19]. It is possibly that the observed functional interaction between GR and PMF-1 is involved in this effect. Nrf-2 is an important transcription factor for regulation of genes involved in antioxidant defense, such as glutathione-*S*-transferase A2 (GSTA2) [30]. Interestingly GR also regulates GSTA2 expression and can repress the Nrf-2 induced activity [31,32]. It is not known whether PMF-1 is found in the Nrf-2 complex on GSTA2 gene promoter as it is on the SSAT promoter. However, our results suggest the possibility that PMF-1 is a cofactor that could be involved in coregulation of GSTA2 by Nrf-2 and GR. Therefore, the observed functional interaction between PMF-1 and GR can be of relevance in GC dependent regulation of cell growth and apoptosis as well as antioxidant defense mechanisms.

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