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Dual roles for lysine 490 of promyelocytic leukemia protein in the transactivation of glucocorticoid receptor-interacting protein 1

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ABSTRACT

Glucocorticoid receptor-interacting protein 1 (GRIP1), a p160 family nuclear receptor co-activator protein, has three activation domains that recruit at least three secondary co-activators: CBP/p300, co-activator-associated arginine methyltransferase 1, and coiled-coil co-activator, which exhibits histone acetyltransferase and/or arginine methyltransferase activities. The regulatory mechanisms underlying the co-activation functions of GRIP1, which associates with promyelocytic leukemia protein (PML) in PML-nuclear bodies, are not well-understood. This study showed that PML specifically and dramatically enhanced the C-terminal transactivation activity of GRIP1 by directly binding to GRIP1 but only when it was sumoylated. Most of the transactivation activity resided in the N-terminal PML regions that are conserved among isoforms. Three N-terminal sumoylation residues (Lys 65, 160, and 490) exhibited differential roles in the regulation of GRIP1 activity, and the sumoylation of Lys 490 acted as the primary nuclear localization signal of PML. While GRIP1 transactivation was stimulated to a similar degree by PML (K490R), located in the nucleus, and wild-type PML, PML (K490D) and the C-truncated mutant PML_{1-489} both displayed an epinuclear localization and were mostly inactive in stimulating GRIP. Based on these data, nuclear foci, nuclear localization, and the sumoylation status of Lys 490 were not essential for the enhancement of GRIP1 activity by PML, but the charge status of Lys 490 was important for subcellular localization of PML and cross-talk between its N- and C-terminal regions to modulate transcriptional activation. Taken together, these results provide insight into the regulatory mechanisms of PML that control the functional activities of GRIP1.

1. Introduction

Glucocorticoid receptor-interacting protein 1 (GRIP1, also known as translation initiation factor 2, TIF2, or steroid receptor coactivator-2, SRC-2) is one of three structurally related but genetically distinct p160 nuclear receptor (NR) co-activators [1–4]. Three activation domains (AD) have been identified in p160: AD1 in the middle region, AD2 in the C-terminal region, and AD3 in the N-terminal region, and each AD can independently activate signal transduction [5–10]. AD1 receives an activating signal from DNA-bound NRs and recruits CREB binding protein (CBP)/p300 [3,6,9] to activate the transcription machinery through its histone acetyltransferase activity. Coactivator-associated arginine methyltransferase 1, a protein with histone methyltransferase activity, mainly binds to the C-terminal region of GRIP1 to stimulate the AD2 transactivation functions [10]. AD3 was recently mapped to the highly conserved N-terminal basic helix–loop–helix/Per–Arnt–Sim domain of p160 co-activators and was shown to recruit secondary co-activators, including coiled-coil co-activator and GRIP1-associated coactivator 63 [5,7].

The most common form of acute promyelocytic leukemia (APL) is characterized by the (15,17) (q21;q22) chromosomal translocation, which leads to the fusion of the gene encoding retinoic acid receptor alpha (RARα) with that encoding promyelocytic leukemia protein

Abbreviations: GRIP1, glucocorticoid receptor-interacting protein 1; TIF2, translation initiation factor 2; SRC, steroid receptor coactivator; NR, nuclear receptor; AD, activation domain; CBP, CREB binding protein; APL, acute promyelocytic leukemia; RAR, retinoic acid receptor; PML, promyelocytic leukemia protein; PML-NB, PML-nuclear body; SUMO, small ubiquitin-like modifier; SIM, sumoylation-interacting motif; Daxx, death-domain associated protein; TSA, trichostatin A; RBCC, a RING-finger, two B-boxes and a predicted α-helical coiled-coil; ATO, arsenic trioxide; atRA, *all-trans* retinoic acid; Ubc9, ubiquitin-conjugating enzyme 9; HA, hemagglutinin; PCR, polymerase chain reaction; DBD, DNA-binding domain; GST, glutathione *S*-transferase; DAPI, 4′,6-diamidino-2-phenylindole; RLU, relative light units; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NLS, nuclear localization signal; Zac1, zinc-finger protein which regulates apoptosis and cell cycle arrest 1

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(PML) [11–13]. The fusion product, PML-RARα, interferes with the normal function of PML and plays an essential role in growth suppression and apoptosis. PML is a tumor suppressor that assembles into a multiprotein nuclear structure to form the PML-nuclear body (PML-NB) and the PML oncogenic domain. PML itself is the principal component of PML-NBs, which have been implicated in the regulation of diverse cellular functions. PML contains a SUMO (small ubiquitin-like modifier) interacting domain (SIM) in its C-terminal region which is important for interaction with nearby sumoylated PML to initiate the nucleation of PML-NBs, as well to promote the recruitment of other PML-NB components such as death-domain associated protein (Daxx), p53, SUMO, and Sp100 [14–17]. While the acetylation of PML by p300 in HeLa cells is potentiated by trichostatin A (TSA) as part of TSA-mediated apoptosis [18], recent studies have shown that the sumoylation of PML in PML-NB formation regulates PML-mediated transcription in three ways, including titration, post-translational modification, and compartmentalization [12,19,20]. The PML protein acts as both a transcriptional co-activator and a co-repressor [21,22], but the contribution of PML-NB to transcriptional regulation is not well understood.

The formation of PML-NBs relies primarily on the self-assembly of the N-terminal RBCC (a RING-finger, two B-boxes and a predicted α-helical coiled-coil) domain of PML and its sumoylation status [23]. Most, but not all PML-NB formation requires covalent SUMO modification or a SIM for targeting PML bodies, as required for SUMO proteases to remove SUMO. A SIM forms a non-covalent interaction with SUMO to attach to its binding partners, the three lysines K65, K160, and K490 in PML, and all isoforms of PML can be sumoylated in vivo [24,25]. The sumoylation of K160 by arsenic trioxide (ATO) subsequently leads to the recruitment of the 11S proteasome, a process that is essential for the degradation of PML and PML-RARα proteins [26]. The SUMO modification of PML finely regulates the dynamics of PML-NB, including ATO- and ring finger protein 4-induced PML degradation [27]. The synergistic therapy of *all-trans* retinoic acid (atRA) and ATO targets two distinct moieties of the oncoprotein PML-RARα, with ATO targeting PML and atRA targeting RARα. ATO degrades both PML-RARα and PML, but not RARα in APL cells [11]. atRA was found to degrade the PML-RARα oncoprotein through a caspase-mediated cleavage pathway and a proteasome-dependent degradation pathway in a biphasic manner [28]. Additionally, atRA increases the level of the cell membrane arsenic channel aquaglyceroporin 9, which allows more arsenic to enter cells [29].

Previous work by Baumann et al. demonstrated that the GRIP1 distribution in the cell is complex and ranges from a diffuse nucleoplasmic pattern to the formation of discrete intranuclear foci. Additionally, they reported that the AD1 region of GRIP1 is associated with the formation of PML-NBs in HeLa cells [30]. While the mechanisms underlying the function of the p160 co-activator in NR transcriptional activation are known, the regulation of p160 by PML is not fully understood. The unique leukemia gene product MOZ-TIF2 may be involved, as it depletes CBP from PML-NBs and leads to the inhibition of p53 and RARβ transcriptional activities [31–33]. This paper presents several lines of evidence demonstrating that PML has functional roles in the regulation of GRIP1 transactivation and NR co-activation, which are mediated, at least partly, by the relief of the repression and self-association phenomena within the GRIP1 C-terminal region via subcellular colocalization.

2. Materials and methods

2.1. Plasmids

Plasmid DNAs encoding pCMV.PML (isoform VI), pCMV.PML(ΔS) and pCMV.PLZF were gifts from Dr. H-M Shih (Academia Sinica, Taipei, Taiwan, ROC) [14] and the plasmids pCMV.PML (isoform IV) and pCMX.HA.PML.RARα were gifts from Dr. H-Y Kao (Case Western Reserve University, USA) [34]. The pSG5.HA (hemagglutinin) vectors encoding various truncated PML fragments were constructed by inserting *Eco*RI–*Xho*I fragments of the appropriate polymerase chain reaction (PCR)-amplified PML isoforms. The pSG5.HA vectors encoding various PML mutants were made by site-directed mutagenesis using the Promega Gene Edit kit (Promega, Madison, MI, USA). The pSG5.HA.RARα vector for human RARα was constructed by inserting *Eco*RI–*Xho*I fragments of appropriate PCR-amplified RARα into the pSG5.HA vector. The pSG5.HA vectors encoding various GRIP1 fragments and Zac1 have been described previously [6,8,35]. Vectors encoding the Gal4DBD (DNA-binding domain) or pM vectors encoding full-length GRIP1 (amino acids 5–1462), GRIP1 (amino acids 5–1121), GRIP1 (amino acids 563–1121), and the GRIP1 C-terminal region (amino acids 1122–1462) have been described previously [6,35]. The pEGFP.C2 vectors encoding various PML and GRIP1 fragments were constructed by inserting *Eco*RI–*Xho*I fragments of the pSG5.HA.PMLs and pSG5.HA.GRIP1s into the *Eco*RI–*Sal*I sites of the pEGFP.C2 vector. The reporter construct GK1 (Gal4 DNA responsive reporter) has been described previously [8].

Bacterial expression vectors for glutathione *S*-transferase (GST) fused to various PML fragments were constructed by inserting the appropriate PCR fragments into the *Eco*RI–*Xho*I sites of pGEX–4T1.

2.2. Cell culture, transient transfection assays, and fluorescence microscopy

HeLa cells were grown in DMEM supplemented with 10% charcoal/dextran-treated fetal bovine serum. Transient transfections and luciferase assays were performed in 24-well culture dishes as described previously [36]. The expression of pEGFP fusion proteins in 24-well culture dishes was observed 16 h after transfection by a fluorescence microscope (Model DMURE2, Leica, Germany) and analyzed with Image-Pro®Plus (Media-cybernetics, USA) [36]. Cells grown on coverslips in 24-well tissue culture dishes were transiently transfected with pSG5.HA vectors encoding various GRIP1 constructs. Cells were fixed in 3.7% formaldehyde for 10 min before permeabilization in PBS containing 0.1% Triton X-100 for 10 min. The antibodies used for immunocytochemistry were: anti-mouse PML (PG-M3; Santa Cruz Biotechnology, USA) and anti-rabbit HA-probe (Y-11; Santa Cruz Biotechnology, USA). The secondary antibodies used for staining were: Alexa Fluor-488 goat anti-mouse IgG (H + L) and Texas Red-X goat anti-rabbit IgG (H + L) (Molecular Probes, Invitrogen, USA). Slides were analyzed by a Leica DMI 6000B Inverted Microscope (Leica Microsystems GmbH, Germany) and images were analyzed with the MetaMorph® System (Molecular Devices, USA). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, Roche, Germany). For luciferase assays, the total DNA for reporter analysis was adjusted to 1 μg by adding the necessary amount of relative empty vector. The luciferase activities of the transfected cell extracts are presented in relative light units (RLU) and expressed as the means and standard deviations of three transfected cultures.

2.3. Protein-protein interaction assays

For GST pull-down assays, ^{35}S-labeled proteins were produced with the TNT T7-coupled reticulocyte lysate system (Promega, USA), and GST fusion proteins were produced in *Escherichia coli* BL21. Radioactively labeled GRIP1 proteins were translated in vitro, incubated with various immobilized GST-PML fusion proteins, eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [9].

2.4. Immunoblot analysis

HeLa cell lysates were prepared in lysis buffer (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100) at 4 °C. Transfected cell extracts were separated by SDS-PAGE, transferred onto a polyvinylidine difluoride membrane (Millipore, USA) and