Daxx has been reported to function as a transcriptional modulator in the nucleus. In the present study, we have explored the role of Daxx in regulating the transcriptional activity of the glucocorticoid receptor (GR). Overexpression of Daxx suppressed GR-mediated activation of the mouse mammary tumor virus promoter in COS-1, HeLa, and 293T cells. In vitro and in vivo studies revealed that Daxx could directly bind to GR. The mapping analysis further demonstrated that the C-terminal region of Daxx-(501–740) mediates the interaction and transcriptional repression of GR. The repressive effect of Daxx and Daxx-(501–740) on GR could be alleviated by co-expression of promyelocytic leukemia protein (PML). Furthermore, immunofluorescence analysis showed that overexpression of wild-type PML results in the translocation of Daxx and Daxx-(501–740) to the PML oncogenic domains (PODs). By contrast, a PML sumoylation-defective mutant failed to recruit Daxx to PODs and to reverse the Daxx repression effect on GR. Accordingly, As2O3 treatment rendered the sequestration of endogenous Daxx to the PODs, leading to an enhancement of GR transactivation in COS-1 cells. Taken together, these findings suggest that recruitment of Daxx into the subnuclear POD structures sequesters it from the GR/co-activators complex, thereby alleviating its repressive effects. Our present studies provide the important link between Daxx/PML interaction and GR transcriptional activation.

The glucocorticoid receptor (GR), a member of the ligand-dependent nuclear receptor superfamily, plays important roles in governing development, growth, apoptosis, and anti-inflammatory processes (1–5). Like other superfamily members, GR contains the N-terminal transactivation domain, central DNA binding domain, and the C-terminal ligand binding domain (6). Prior to exposure to its respective ligand, the GR and other receptors in this family form complexes with heat shock proteins (HSPs), restricting the unliganded-receptor in its inactive state. Upon ligand binding, the receptor is dissociated from HSPs and translocates to the nucleus where the liganded receptor binds to the glucocorticoid-responsive element and recruits co-factors to regulate transcription (7). A diverse group of proteins have emerged as potential cofactors for nuclear receptors through direct protein-protein interactions. For instances, several co-activators have been identified that bind to GR and modulate GR transcriptional activity, including CBP/p300, p160 family co-activators (SRC-1, GRIP1, and p/CIP), and chromatin remodeling complexes (BRG-1 (SWI/SNF) complex and P/CAF (ADA/SAGA) complex) (8, 9). These co-activators appear to be assembled in a multiprotein complex, facilitating the access of nuclear receptors and the RNA polymerase II core machinery to their target DNA by a chromatin remodeling process (10).

In addition to regulating GR transcriptional activity via direct protein-protein interactions, some co-factors appear to modulate GR activity through indirect fashion. PML, a protein located at the promyelocytic oncogenic domains (PODs), has previously been reported to stimulate GR and RXR transcriptional activity through its interaction with CBP (11). These results demonstrated that a portion of CBP is recruited to the PODs through its association with PML, suggesting that PML and/or POD-associated proteins may function as an important cofactor in governing nuclear hormone receptor transcriptional activity and function. Besides PML and CBP, several cellular proteins have been reported to be associated with PODs, including Sp100, Daxx, p53, Rb, small ubiquitin-like modifier (SUMO-1), and BLM (12, 13). Results from many elegant studies have revealed that overexpression of p53 suppressed GR-mediated reporter gene activation (14, 15) and Rb potentiated GR transcriptional activity through its interaction with hBRM (16), respectively. Recently, SUMO-1 modification of GR has been shown to regulate its transcriptional activity (17, 18). These findings suggest that POD-associated proteins play important roles in modulating GR transcriptional activity. Whether other POD-associated proteins are also involved in regulating GR transcriptional activity remains to be explored.

Daxx, a PODs-associated protein, was identified as an interacting protein of PML (19–21). Recent studies implied that...
Daxx might function as a transcriptional coregulator. Daxx has been shown to exhibit transcriptional repression activity by inhibiting the transcription factor Pax3 and ETS1 through direct protein-protein interactions (22, 23). Most recently, Emelyanov et al. reported that Daxx acts as a transcriptional co-activator or co-repressor of Pax5 in different cellular contexts (24). Although the exact mechanism accounting for these observations is still unclear, the recruitment of nuclear factors possessing either histone acetyltransferase or histone deacetylation activity by Daxx to modulate Pax5 transcriptional activity was proposed (24). In addition, the transcriptional repression effect of Daxx could be modulated by subnuclear compartmentalization through protein-protein interactions. We have recently demonstrated that overexpression of a nucleolar protein MSP58 alleviates the transcriptional repression elicited by Daxx, correlating well with sequestration of Daxx to the nucleolus via Daxx/MSP58 protein-protein interactions (25). Furthermore, Lehembre et al. has shown that SUMO-1-modified PML could relieve the transpression effect of Daxx on Pax3 transcriptional activity through sequestering Daxx into the PODs (26). Taken together, PML may exert its regulatory effect on transcription factor(s) through altering the compartmentalization of Daxx or other co-regulator(s). This notion led us to raise a question as to whether GR is also regulated by PML, at least in part, through Daxx sequestration to the PODs.

In the present study, we have characterized both biochemical and functional interactions between Daxx and GR. Furthermore, the Daxx-mediated GR repression could be alleviated by the co-expression of wild-type PML but not by a sumoylation-defective PML mutant, which also fails to recruit Daxx to the PODs. Our findings thus provide the first evidence that Daxx could modulate the transcriptional activity of nuclear hormone receptor through protein-protein interactions and sheds light on the mechanism underlying the PML role in modulating nuclear hormone receptor function.

EXPERIMENTAL PROCEDURES

Plasmids Construction—LexA-Daxx, its deletion mutants LexA-MST3, LexA-lamin, HA-Daxx, and its derived mutant constructs have been described before (25). A mammalian GST-Daxx expression vector was constructed by inserting Daxx into pDEST26 (Invitrogen). MMTV-Luc and pCMX-PML were kind gifts from Drs. Chawness Chang and Ronald Evan, respectively. pGal-AD-GR was engineered by inserting GR coding region into the BamHI and XhoI sites of yeast vector pACT2, which expresses the Gal4 activation domain (Clontech). pCMX-PML-ΔSUMO contains a lysine-to-arginine mutation at amino acid positions 65, 160, and 490 was created in three rounds consecutively of site-directed mutagenesis on the same template pCMX-PML. Site-directed mutagenesis was conducted by using QuikChange site-directed mutagenesis kit according to the manufacturer’s instruction (Stratagene).

Two-hybrid Assay and β-Galactosidase Assay—Yeast two-hybrid assays were performed as described (25). Briefly, L40 yeast strain was first transformed with individual bait and followed by the prey construct transformation. Yeast transformants were selected on medium lacking histidine, leucine, and tryptophan for four days. His+ colonies were further tested for β-galactosidase activity. Quantitative X-gal assays were performed with yeasts containing pairs of bait and prey plasmids as indicated. The X-gal activities were determined from three separate liquid yeast cultures according to the instructions of the β-galactosidase assay, correlating well with sequestration of Daxx to the nucleolus via Daxx/MSP58 protein-protein interactions (25).

Immunoprecipitation and Western Blotting Assays—For purification of GST and GST-Daxx fusion proteins, 293T cells were transfected with pDEST26 (Invitrogen) or pDEST26-Daxx. Cells were lysed 48 h later in buffer EM2 containing 50 mM HEPES (pH 7.6), 50 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10% glycerol, and 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) and centrifuged at 17,000 × g for 10 min at 4 °C. Supernatants were incubated with glutathione-agarose beads for 1 h at 4 °C. The bound proteins were then analyzed by SDS-PAGE followed by Western blotting using antibody against GST protein. 35S-labeled GR protein was made with the TNT reticulocyte lysate system (Promega). 35S-labeled proteins were incubated with GST or GST-Daxx-agarose beads, which contained equal amounts of protein in 0.2 ml of binding buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 0.5 mM EDTA) for 2 h, washed four times, and analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation and Western Blotting Assays—Yeast transformants were selected on medium lacking histidine, leucine, and tryptophan for four days. His+ colonies were further tested for β-galactosidase activity. Quantitative X-gal assays were performed with yeasts containing pairs of bait and prey plasmids as indicated. The X-gal activities were determined from three separate liquid yeast cultures according to the instructions of the β-galactosidase assay, correlating well with sequestration of Daxx to the nucleolus via Daxx/MSP58 protein-protein interactions (25).

RESULTS

Overexpression of Daxx Suppresses GR-mediated Transactivation of the MMTV Promoter—To test whether Daxx is involved in regulating GR transcriptional activation, the Daxx expression construct was cotransfected into COS-1 cells along with the GR expression construct and the MMTV-Luc reporter. As shown in Fig. 1A, Daxx suppressed GR-mediated transactivation in a dose-dependent fashion. By contrast, a transcriptional co-activator, GRIP1, potentiated the transactivation ability of GR. Furthermore, the repressive effect of Daxx on GR was also observed in other cell lines, such as HeLa and 293T cells (Fig. 1B), indicating that the Daxx-dependent GR repression is not a cell type-specific event.

Daxx Binds to GR Both in Vitro and in Vivo—To explore whether the transcriptional repression of GR by Daxx is through the direct protein-protein interaction between Daxx and GR, we first performed the co-immunoprecipitation experi-
COS-1 cells were cotransfected with expression constructs encoding GR and HA-Daxx and followed by treatment of dexamethasone. Forty-eight hours after transfection, cell lysates were subjected to immunoprecipitation assays with an anti-GR antibody followed by Western blot analyses with an anti-HA antibody. As shown in Fig. 2A, Daxx was detected in the immunoprecipitated complexes of GR (top panel, lane 4). This interaction was also validated in a reciprocal co-immunoprecipitation assay (data not shown). These results suggest that Daxx and GR can associate to form complexes in mammalian cells.

To further test whether this association is through direct

FIG. 2. Interaction between GR and Daxx in vitro and in vivo. A, GR interacts with Daxx in transfected COS-1 cells. COS-1 cells were transiently transfected with the indicated combinations of expression constructs encoding GR and HA-tagged Daxx, respectively. Following treatment with 100 nM Dex for 30 h, total cell lysates were analyzed by Western blot (WB) to check expression of GR and Daxx proteins (bottom panel). Equal amounts of cell lysates were immunoprecipitated (IP) with an anti-GR antibody, followed by blotting with an anti-HA antibody (upper panel), whereas reciprocal co-immunoprecipitation experiments were performed using an anti-HA antibody, followed by blotting with an anti-GR antibody (data not shown). B, interaction of GST-Daxx with in vitro translated GR. The GST or GST-Daxx fusion proteins were immobilized on the glutathione-agarose beads and then incubated with in vitro translated [35S]methionine-labeled GR protein. After wash, the bound components were resolved on a 10% SDS-polyacrylamide gel. The 20% amount of input GR was shown as a positive control for GR. Immunoblotting analysis of GST and GST-Daxx protein using an antibody against GST were aligned to show protein levels (bottom panel).
protein-protein interaction, GST pull-down assays were performed using GST-Daxx fusion protein and in vitro translated [35S]methionine-labeled GR. As shown in Fig. 2B, GR was specifically pulled down by GST-Daxx but not by GST protein, indicating that GR binds to Daxx directly. It should be noted that the interaction between GR and Daxx was not affected by the presence or absence of dexamethasone. To further substantiate the observed direct protein-protein interaction between Daxx and GR, we also carried out the yeast two-hybrid assays. The GR cDNA clone was subcloned in frame into pACT2 vector (Gal4-AD) and subsequently analyzed with different bait proteins (Daxx, MST3, and lamin) for the ability to activate His3 and LacZ reporter genes. As summarized in Fig. 3B, yeast co-transformed with GalAD-GR and LexA-Daxx was able to form colonies in the medium plate lacking histidine, indicating a positive interaction between GR and Daxx. Again, this interaction is specific since no interaction between GR and lamin or MST3 was detected. The interaction was further verified by liquid β-galactosidase assay (Fig. 3C). Taken together, our results clearly demonstrated that GR interacts with Daxx through direct protein-protein interactions.

The Functional Interaction between the C-terminal Domain of Daxx and GR—To delineate the region(s) of Daxx that is involved in Daxx/GR interaction, various deletion constructs of Daxx were engineered (Fig. 3A) and subjected to analyses in yeast two-hybrid assays. The strength of interaction was scored by the colony growth in histidine-auxotroph-selective medium and quantified by liquid β-galactosidase assay. As shown in Fig. 3B, the C terminus-deleted Daxx (amino acids 1–501 and 1–625) failed to interact with GR. In contrast, the mutant with a deletion of amino acid residues 1–501 of Daxx was still capable of interacting with GR, albeit the strength of interaction with GR was less than that from the full-length Daxx. Additionally, consistent results were also observed with the liquid β-galactosidase assay. Altogether, these results implicated that the C-terminal region of Daxx is necessary and sufficient for its interaction with GR.

To establish the correlation between interaction and GR transcriptional repression by Daxx, these Daxx mutants were analyzed for their respective abilities to repress GR transcription in mammalian cells. As illustrated in Fig. 3D, overexpression of Daxx-(501–740), like full-length Daxx, was capable of suppressing GR-mediated transcriptional activation, whereas both Daxx-(1–501) and Daxx-(1–625) failed to do so, indicating that the
C-terminal region of Daxx is sufficient for Daxx-mediated GR transrepression. Taken together, these findings provide direct evidence that the interaction between Daxx and GR correlates well with Daxx function as a GR transrepressor.

**PML Reverses Daxx Transrepression of GR by Recruiting Daxx to the PODs**—Recent studies indicated that PML overexpression reverses the Daxx-dependent transcriptional repression and such a de-repression correlates well with the ability of sumoylated PML to sequester Daxx to the PODs (21). In this scenario, we anticipated that PML should be able to relieve the Daxx transrepression on GR activity. To test this possibility, COS-1 cells were cotransfected with PML expression construct along with the expression constructs harboring either full-length Daxx or Daxx(501–740) and assayed for GR transcriptional activity. As shown in Fig. 4A, overexpression of PML resulted in a reduction of transrepression elicited by both Daxx and Daxx(501–740) on GR in a dose-dependent manner. To further substantiate that the recruitment of Daxx by PML plays an important role in regulating GR transcriptional activity, a PML sumoylation-defective mutant was subjected to the same analyses for its ability to recruit Daxx to the PODs (Fig. 5A). As expected, sumoylation-defective PML mutant, unlike the wild-type PML, was unable to relieve the Daxx transrepression and the recruitment of Daxx to PODs by PML (Fig. 5B), indicating that the Daxx-mediated GR transrepression and the recruitment of Daxx to PODs by PML are an interdependent event. To further demonstrate the effect exerted by PML on the Daxx-dependent GR transrepression and the Daxx nuclear compartmentalization, we treated COS-1 cells with As$_2$O$_3$. As$_2$O$_3$ has been shown to promote the SUMO-1 modification of PML, which in turn induces the re-

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*D.-Y. Lin and H.-M. Shih, unpublished data.*
cruitment of PML and Daxx to the PODs (19, 21, 26). As shown in Fig. 6A, As2O3 treatment caused a marked increase of the number of POD formation, as evidenced by immunofluorescent analysis of endogenous PML (a and d). This treatment also rendered the redistribution of endogenous Daxx from nucleoplasm to the PODs, leading to the co-localization of Daxx with PML (b and e). Coordinately, As2O3 treatment resulted in an increment of the GR-mediated transcriptional activity (Fig. 6B). Taken together, the results of transient cotransfection assays and immunofluorescence studies unequivocally indicate that PML potentiates GR transcriptional activation by compartmentalizing Daxx from nucleoplasm to the PODs.

**DISCUSSION**

In the present studies, we have identified Daxx as a GR-interacting protein and demonstrated that Daxx regulates the GR transcriptional activation on MMTV promoter. We provided biochemical evidence that Daxx associates with GR in yeast, *in vitro*, and in mammalian cells. The interaction between GR and Daxx is mediated through the C-terminal region of Daxx. Furthermore, co-expression of PML relieves the transcriptional repression by Daxx, correlating well with the recruitment of Daxx from the nucleoplasm to the PODs. Our findings not only provide the first direct link between Daxx expression and GR activity but also shed some light on the molecular mechanism underlying the role of PML in regulating GR transcriptional potential.

Daxx was initially identified as the adaptor molecule between Fas receptor and Jun N-terminal kinase in Fas-mediated apoptosis (27). However, accumulated evidence suggested that Daxx might function as a transcriptional repressor through its interaction with a growing number of transcription factors. Daxx has been shown to repress the basal activity by a heterologous TK promoter through Gal4DBD-Daxx fusion (19, 21, 25). Daxx was also reported to inhibit the activity of the transcriptional activators Pax3 and ETS1 (22, 23). While Daxx overexpression inhibited the endogenous Pax5 transcriptional activity in the human HS-Sultan cell line and the murine plasmacytoma J558L cell line, Daxx potentiated Pax5 transcriptional activity in M12.4.1 and A20 murine B cells (24). In the present study, we further demonstrated that Daxx could suppress the GR-mediated transcriptional activation of MMTV reporter. The transrepressional effect of Daxx on GR activity has also been demonstrated in several cell lines, indicating that this repressive effect is likely not a cell type-specific event. Furthermore, we also provided biochemical and cell biological evidence that the C-terminal region of Daxx is necessary and sufficient to interact with GR and repress GR transcriptional activity. Intriguingly, this C-terminal region of Daxx has also been reported to interact with other transcription factors, such as Pax3, Pax5, and ETS1 (19, 23, 24) as well as a wide variety of molecules, including Fas receptor, PML, centromeric protein CENP-C, DNA methyltransferase 1, HSP27, Glut4, Ubc9, and SUMO-1 (21, 27–32). Conceivably, the C-terminal region of Daxx acts as a major docking domain for protein-protein inter-
actions. To our knowledge, it is not yet demonstrated how this domain provides a binding surface for these proteins with such distinctive functions. However, we postulate that Daxx is likely involved in the signaling pathway cross-talks through competitive binding by distinct factors to the specific motif(s) located within the C-terminal domain of Daxx.

Although Daxx functions as a transcriptional co-repressor, the molecular mechanism as to how it suppresses the transcriptional activation remains largely unknown. Previous report showed that the treatment with a deacetylase inhibitor, trichostatin A, efficiently reversed the repressive effect of Daxx (21), suggesting that histone deacetylation may be involved in Daxx-mediated transcriptional repression. Consistently, interaction between the Daxx and HDAC1 has been demonstrated by in vitro pull-down assay and in vivo overexpression experiments (21). Furthermore, Hollenbach et al. has recently shown that endogenous hDaxx eluted from size exclusion chromatography column has an apparent molecular mass of 360 kDa and associates with multiple proteins that are critical for transcriptional repression, such as histone deacetylase II, components of chromatin such as core histone H2A, H2B, H3, and H4, and a chromatin-associated protein Dek (33). The findings of the hDaxx association with histone deacetylase are consistent with our notion that HDACs play an essential role in Daxx-mediated transcriptional modulation via histone remodeling processes. Moreover, that Daxx was reported to be associated with condensed chromatin in the cells lacking PML (20) also supports the role of Daxx in the establishment and/or maintenance of a transcriptionally silenced chromatin structure. Whether overexpressed Daxx results in silencing the chromatin structure of GR-regulated genes remains to be explored.

Another main feature of the present study is to explore the mechanism as to how PML regulates GR transcriptional activity. PML has been proposed to function as a transcription cofactor through recruiting co-activators (13, 34). For example, PML has been shown to potentiate the transcriptional activity of AP-1 and progesterone receptor (35, 36). Further studies revealed that PML does not interact directly with either Jun/Fos or progesterone receptor but instead targets transcriptional co-activators, such as CBP and TIF1α (11, 36, 37). PML binds to CBP in vitro, promotes CBP localization to PODs, and enhances transcriptional activity of nuclear receptors such as GR and retinoic acid receptor in transient transfection assays (11). Similarly, a co-activator complex, composed of PML, TIF1α, and CBP, is recruited to retinoic acid response element, suggesting that PML is a co-activator for retinoic acid receptors (37). In this study, we propose an alternative regulatory route for PML in modulating GR transcriptional function. The recruitment of Daxx to PODs by PML may represent another mechanism whereby the activity of the GR is activated. Our results clearly demonstrated that wild-type but not sumoylation-defective PML is critical for attenuating transrepression effect of Daxx on GR by translocating Daxx to the PODs. Likewise, that As2O3-treatment enhanced GR transcriptional activation utilizes a similar mechanism by modulating Daxx localization. These findings are consistent with the established notion from recent studies that SUMO-1 modification of PML is required for both the sequestration of Daxx to the PODs and efficient inhibition of Daxx-mediated repression. One may hypothesize that retention of Daxx into PODs through PML overexpression would prevent the access of Daxx to GR, subsequently impeding its repressive action. Likewise, Lehembre et al. have recently reported that SUMO-1-modified PML could derepress Pax3 transcriptional activity through sequestration of the Daxx repressor into the PODs (26). Taken together, these findings suggest that PML may modulate the activities of a variety of transcription factors by altering the compartmentalization of its respective co-repressor(s) to the PODs. Comparable to this scenario, we have recently identified that nuclear protein MSP58 can enhance the transcriptional potential by sequestering Daxx to the nucleolus (25). In light of these observations, PODs and/or nucleolus may participate to the transcriptional activation of specific target genes by tethering corepressors, such as Daxx, from the diffuse nuclear fraction where transcription takes place.

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