Essential Role of the 58-kDa Microspherule Protein in the Modulation of Daxx-dependent Transcriptional Repression as Revealed by Nucleolar Sequestration*

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Daxx has been reported to mediate the Fas/JNK-dependent signals in the cytoplasm. However, several lines of evidence have suggested that Daxx is located mainly in the nucleus and functions as a transcriptional regulator. Recent studies have further indicated that Daxx-elicited transcriptional repression can be inhibited by the nuclear body-associated promyelocytic leukemia protein and apoptosis signal-regulating kinase 1 by sequestering Daxx to the nuclear bodies and the cytoplasm, respectively. Here, we further investigated the coordinated molecular mechanism by which Daxx function is regulated through protein-protein interaction. Using yeast two-hybrid screens to identify Daxx-interacting protein(s), three independent clones encoding the 58-kDa microspherule protein (MSP58) fragments were identified. Furthermore, we have demonstrated that Daxx interacts in vitro and in vivo with MSP58 via its NH2-terminal segment, which is distinct from the binding region of Fas, apoptosis signal-regulating kinase 1, and promyelocytic leukemia protein, suggesting a unique regulatory role of MSP58 on Daxx function. Transient transfection experiments revealed that MSP58 relieves the repressor activity of Daxx in a dose-dependent manner in COS-1 and 293 cells but not in HeLa cells, implicating cell type-specific modulation of Daxx function by MSP58. Moreover, immunofluorescence analysis unequivocally demonstrated that MSP58 overexpression results in a translocation of Daxx to the enlarged nucleoli in COS-1 or 293 cells, whereas Daxx exhibited a diffuse nuclear pattern in HeLa cells. Taken together, these findings delineate a network of regulatory signaling pathways that converges on MSP58/Daxx interaction, causally associating Daxx nucleolus targeting with its transcriptional activation function.

Daxx was initially identified in yeast two-hybrid screens as a protein associated with the death domain of the Fas receptor and was thought to be involved in promoting Fas-induced apoptosis (1). The overexpression of Daxx, notably in cooperation with Fas overexpression, resulted in an enhanced Fas-mediated apoptosis and the activation of Jun NH2-terminal kinase (JNK)3 pathway (1). Previous reports have demonstrated that the COOH-terminal portion of Daxx is involved in its interaction with Fas receptor (1). Moreover, the overexpression of the Daxx COOH-terminal domain inhibited both Fas-induced JNK activation and Fas-induced apoptosis (1). To further elucidate the role of Daxx in the apoptosis process, Chang et al. (2) has reported that Daxx-induced JNK pathway activation appears through a direct protein-protein interaction and apoptosis signal-regulating kinase 1 (ASK1) kinase activation. The overexpression of a kinase-deficient ASK1 was shown to inhibit Fas-and Daxx-induced apoptosis. Taken together, these findings suggested that Daxx functions as an adaptor protein linking Fas signaling to JNK pathway via ASK1. Furthermore, upon stimulation of the death receptor Fas, Daxx translocates from the nucleus to the cytoplasm and triggers caspase-independent cell death by association with ASK1 (2, 3). Recently, two reports (4, 5) have demonstrated that ASK1 activation is required for Daxx to mediate Fas-induced signaling and localize in the cytoplasm. In addition, heat shock protein 27 has been reported to interact with Daxx to prevent Fas-induced Daxx translocalization from the nucleus to the cytoplasm, leading to the inhibition of Fas-induced Daxx- and ASK1-dependent apoptosis (3). Hence, these observations have suggested a role for Daxx in a Fas-mediated signal transduction pathway and as a pro-apoptotic signal mediator in the cytoplasm. Moreover, Daxx has also been reported to be associated directly with the cytoplasmic domain of the type II tumor growth factor-β receptor, mediating tumor growth factor-β-induced apoptosis and JNK activation (6). In this respect, Daxx is postulated to function in the cytoplasm as a signal transducer between the cell-surface receptor and the ASK1/JNK kinase cascade. However, several lines of evidence also indicate that Daxx may play a direct role in the nuclear events to modulate gene expression. In particular, several groups (1, 2, 7) have reported that Daxx is present exclusively in the nucleus, a result that appeared inconsistent with the findings that Daxx is part of a cytoplasmic multiprotein complex of Fas, ASK1, and Daxx upon Fas stimulation. Furthermore, it has been reported that Daxx interacts with SUMO-modified PML, which serves as a component of nuclear domain 10, also referred to as the PML bodies or PML oncogenic domains (PODs) (8–10). The results

* This work was supported by intramural funds of National Health Research Institutes (to H.-M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: JNK, c-Jun NH2-terminal kinase; ASK1, apoptosis signal-regulating kinase 1; SUMO, small ubiquitin-like modifier; PML, promyelocytic leukemia protein; ETS, E26 avian leukemia oncogene 1; MST3, mammalian STE20-like kinase 3; DBD, DNA-binding domain; MSP58, 58-kDa microspherule protein; GST, glutathione S-transferase; HA, hemagglutinin; GR, glucocorticoid receptor; Dex, dexamethasone; PODs, PML oncogenic domains; PAH, paired amphipathic helices.
from these studies led to a hypothesis that the Daxx enhancement of Fas-induced apoptosis, at least in part, is a consequence of PML-mediated association of Daxx with PODs in the nucleus (8). Hence, it is possible that the interaction between PML and Daxx constitutes a novel nuclear pathway to elicit apoptotic response (11). Additional supports for a nuclear function of Daxx came from reports on the identification of its nuclear interacting proteins as well as its intriguing property to repress basal and activated transcription (8, 12, 13). Daxx can inhibit the activity of the transcriptional activator ETS1, leading to the down-regulation expression of two ETS1 target genes, MMP1 and Bcl-2 (13). In addition, Daxx has been shown to interact with DNA methyltransferase 1, implicating its role in gene silencing (14). However, the mechanism for the Daxx-mediated repression remains to be unraveled, but its interaction with the histone deacetylase 1 implies that histone deacetylation is potentially involved in the trans-repression events (10). Several studies revealed that effective sumoylation of PML and subsequent recruitment to PODs modulate transcriptional activity of Daxx. Upon sumoylation of PML, the Daxx-mediated transcriptional repression is attenuated, whereas such a derepression correlates well with the sequestration of Daxx to the PODs (10). Moreover, Lehembre et al. (15) have demonstrated that SUMO-1-modified PML can derepress Pax3 transcriptional activity through the sequestration of Daxx into the PODs. Taken together, these observations suggest that Daxx functions as a transcriptional repressor whose repressive effect can be modulated by PML through the subnuclear compartmentalization.

Protein shuttling within the individual cell may play an important regulatory role for protein functions. Accumulated evidence has suggested that Daxx can be shuttled within subnuclear compartments. For example, sumoylated PML is able to interact with Daxx, leading to the recruitment of Daxx to the compartment of PODs. Furthermore, Daxx was found to interact with the centromere protein C (CENP-C), implicating an interphase-restricted association of Daxx with centromeres (16). The results from immunofluorescence studies have revealed that there is a dynamic and cell cycle-regulated shuttling of Daxx between centromeres and PODs (16, 17). In addition, the translocation of Daxx to PODs is also involved in interferon-triggered apoptosis (18), suggesting that intracellular protein localization is tightly regulated in cells. Although the functional significance of these observations remains to be established, it is highly likely that a regulatory process governing protein trafficking exists in response to a variety of physiological and pathological alterations in many different cell types.

To explore the nuclear factor(s) that modulates Daxx function, we have searched the Daxx-interacting proteins using a yeast two-hybrid library screen. In this study, we have characterized both biochemical and functional interactions between Daxx and MSP58, a nucleolar protein that has been identified as a p120-interacting protein (19). The overexpression of MSP58 relieves the transcriptional repression mediated by Daxx. Furthermore, we show that the Daxx-mediated repression of glucoctocytocin receptor (GR) transcriptional activity can be inhibited by MSP58. Intriguingly, such a derepression correlates well with the sequestration of Daxx to the nucleolus, which is mediated through its interaction with the MSP58. The mechanism for regulating Daxx function is comparable with PML sequestration of Daxx to the PODs. Our findings reveal a novel regulatory role for the nucleolar structure and nucleolar protein, MSP58, in modulating Daxx-mediated transcriptional repression.

**EXPERIMENTAL PROCEDURES**

**Two-hybrid Screen and β-Galactosidase Assay**—The DNA fragments encoding the full-length or a portion of human Daxx were generated by polymerase chain reaction and subsequently inserted into the pBTM116 vector to produce baits for yeast two-hybrid studies. The LexA-Daxx construct was used to screen against with human testis cDNA library (CLONTHEC). Yeast two-hybrid screen was performed as previously described (20). L40 yeast strain was first transformed with pLexA-Daxx and followed by 250 μg of the testis cDNA library transformation. The library transformants were selected on medium lacking histidine, leucine, and tryptophan. His+ colonies were further tested for β-galactosidase activity using a colony lift filter assay. The plasmids from both His+ and X-gal+ colonies were isolated by the curing process of MC1066 bacterial strain and retransformed with LexA-Daxx, LexA-MST3, or LexA-lamin to test the binding specificity. The library plasmids conferred that the Daxx-specific interactions were then subjected to DNA sequence analysis. 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 Galacto-light Plus kit (Tropix Inc, Bedford, MA).

**Plasmid Construction**—A PCR fragment encoding full-length (amino acids 1–740) of human Daxx was subcloned into vector pBTM116 in-frame with LexA to generate the LexA-Daxx bait. PML and MST3 were also fused in-frame with LexA in pBTM116. A series of Daxx deletion fragments were inserted into pBTM116 and pcDNA3-HA vectors for fusion protein expression in yeast and mammalian cells, respectively. To obtain pACT2-MSP58, pCMV-FLAG-MSP58, and pG5-MSP58, the full-length clone of human testis cDNA library and inserted into the vector pACT2, pCMV-tag2 (Stratagene), or pGEX-5X-2 (Amersham Biosciences), respectively. pG5-luciferase with five Gal4 binding sites in front of the minimal promoter driving the luciferase reporter gene is from Stratagene. An SV40-driven β-galactosidase reporter plasmid, pSVβ-GAL, was used in this study as an internal control for transfection efficiency. Gal-DBD fusion proteins containing full-length Daxx and Daxx fragments were generated by PCR and cloned into the BamH1 site of pCMX-Gal-DBD plasmid. Mammalian vectors expressing PML and GR were gifts from Dr. Ronald M. Evans and Dr. David Ann, respectively. The mouse mammary tumor virus long terminal repeat-luciferase reporter construct was generously provided by Dr. Chawnessh Chang.

**GST Pull-down Assay**—The expression and purification of GST fusion proteins were performed as described previously (21). Various 35S-labeled Daxx proteins were made with in vitro transcription and translation reticulocyte lysate system (Promega). 35S-labeled proteins were incubated with 2 μg of each GST-MSP58 fusion 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 1–2 h, washed four times, and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining to visualize GST fusion proteins.

**Immunoprecipitation and Western Blotting**—For testing the association in mammalian cells, various HA-Daxx and FLAG-MSP58 constructs were transfected into CO-S1 cells 70% confluent in a 10-cm dish. Thirty-six hours after transfection, cells were solubilized in 1 ml of lysis buffer (50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 15 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, and 0.1% sodium deoxycholate and protease inhibitor mixture (Complete, Roche Molecular Biochemicals)). Whole cell lysates were mixed with antiserum against HA (BABCO, Richmond, CA) or against FLAG M2 antibody (Sigma), and the immunocomplexes were mixed with protein A-Sepharose beads (Amersham Biosciences). After 2 h of incubation, the immunocomplexes were then gently washed three times with the same buffer as described above, followed by Western blot analysis with the anti-HA antibody or with the anti-FLAG antibody. Proteins were detected using the ECL kits (Amersham Biosciences). Anti-Gal-4DBD antibody was purchased from Santa Cruz Biotechnology.

**Immunofluorescence**—The monkey COS-1, HeLa, and 293 cells was transfected with pcDNA3-HA-Daxx, pCMX-PML, or pCMV-FLAG-MSP58 by the lipofection method. Forty-eight hour after transfection, the cells were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline and then permeabilized with 0.4% Triton X-100. The cells were then incubated with anti-FLAG M2 antibody (Sigma), anti-PML monoclonal antibody (PG-M3, Santa Cruz Biotechnology), or anti-HA polyclone (Y-11, Santa Cruz Biotechnology) for 1 h at room temperature and washed three times with phosphate-buffered saline followed by incubation with fluorescein-conjugated anti-mouse IgG.
**RESULTS**

Identification of MSP58 as a Daxx-interacting Protein—To identify Daxx-interacting proteins, we have performed a yeast two-hybrid screen using the full-length of human Daxx fused to the LexA protein, LexA-Daxx, as bait. A plasmid library constructed by fusing the transcription activation domain to the individual cDNA prepared from human testis was screened for the interaction between LexA-Daxx and its interacting proteins in the yeast L40 reporter strain. A total of 10^6 transformants were screened by a selection for His prototrophy on yeast drop-out medium lacking histidine. Positive clones were confirmed by the expression of β-galactosidase in X-gal filter assays. Among the 87 His+ and β-galactosidase positive clones examined, three independent clones (amino acids 15–50, 5–46, and 14–22) were identified to encode for different but overlapping portions of an identical protein. Sequence analyses revealed that the inserts from these individual clones corresponded to the NH2-terminal region of MSP58 protein (19). A schematic representation of the MSP58 protein along with the clones interacting with LexA-Daxx is shown in Fig. 1A, top panel. The interactions between the fusion protein encoded by these three results are presented as the means ± S.E. of at least three independent experiments.

**Identification of MSP58 as a Daxx-interacting Protein**

**Cell lines, Transfection, and Reporter Gene Assay**—All mammalian cell lines were obtained from the American Type Culture Collection (Manassas, VA). COS-1 and 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Cells were seeded into 10-cm plates the day before transfection. Transient transfections were carried out using LipofectAMINE transfection kit (Invitrogen). Cell extracts were harvested 36 h later for co-immunoprecipitation assays and Western blot analysis. For the reporter gene assay, 2–3 × 10^6 cells were seeded on 6-well plates 24 h prior to transfections, and 4 h before transfection, the cells received fresh medium with 10% fetal bovine serum. Approximately 2 µg of total plasmid DNA/well were used in the transfection. The total amount of plasmid per well was kept constant by adding pcDNA3 empty vector. Cells were harvested 36 h later, and cell extracts were assayed for luciferase activity (Packard, Meriden, CT). For the experiments of GR induction, cells were induced with dexamethasone (Dex) 18 h after the start of transfection and then harvested after an additional 24 h. Luminescence was measured on a top counter microplate scintillation and luminescence counter (Packard 9912V) in single photon counting mode for 0.1 min/well following a 10-min adaptation in the dark. Luciferase values were normalized by the internal control β-galactosidase activity. Experiments were performed in triplicate. Experimental procedures, and values are given as "Experimental Procedures."
clones and Daxx were specific, because other fusion proteins such as LexA-lamin or LexA-MST3 (a serine/threonine kinase) did not confer the same magnitude of /H9252-galactosidase activity elicited by LexA-Daxx as determined by the quantitation X-gal assay of transformed yeasts (Fig. 1A, bottom panel). MSP58 protein has been reported to interact with the proliferation-related nucleolar protein p120 (19). MSP58 contains unique NH2-terminal serine-rich clusters and the COOH-terminal region with a coiled coil domain. The COOH-terminal region of MSP58 is required for binding to p120 by domain-mapping studies (19). Thus, the specific and distinct interaction of Daxx with the fusion protein of these three isolated clones indicated that Daxx binds to the NH2-terminal region spanning amino acids 1–291 of MSP58, which is distinguished from the previously reported p120-interacting domain.

To further verify the protein interaction between Daxx and full-length MSP58 in yeast, the full-length MSP58 cDNA was amplified by PCR using specific primer pairs designed that correspond to the nucleotide sequence of human MSP58. The derived MSP58 cDNA was subcloned in-frame into the pACT2 vector, GalAD-MSP58, and subsequently analyzed with different bait proteins (lamin, MST3, Daxx, and PML) for the ability to activate HIS3 and lacZ reporter genes (Fig. 1B). Yeast co-transformed with GalAD-MSP58, and LexA-Daxx was able to form colonies in the medium plate lacking histidine, indicating a positive interaction between MSP58 and Daxx. Again, the interaction is specific, because no interaction was detected between MSP58 and lamin or MST3 or PML, whereas the interactions between SUMO-1 and Daxx and PML served as positive controls. The interaction was further verified by liquid β-galactosidase assay (Fig. 1B, bottom panel). Taken together, our results clearly demonstrated that MSP58 interacts with Daxx in a specific manner in yeast.

Interaction of MSP58 with Daxx NH2-terminal in Yeast and in Vitro—To delineate the region(s) of Daxx, which is involved in MSP58-Daxx interaction, various deletion constructs of Daxx were engineered and subjected to analyses in yeast two-hybrid assay. The strength of interaction was quantified by liquid β-galactosidase assay. As shown in Fig. 2, the COOH-terminus-deleted Daxx (amino acids 1–501 and 1–625) interacted with MSP58 approximately 2- and 3-fold, respectively, more strongly than that conferred by the full-length Daxx. In contrast, the deletion of amino acid residues 1–501 of Daxx completely abolished its interaction with MSP58. These results implicated the NH2-terminal region of Daxx as being sufficient for stable interaction with MSP58.

To further confirm these observed interactions in vitro, GST pull-down experiments were carried out using GST-MSP58 fusion protein and a battery of in vitro translated [35S]methionine-labeled Daxx and various deletion mutants. As shown in Fig. 2B, the full-length Daxx was specifically pulled down by GST-MSP58 but not by GST protein. In agreement with the results of yeast

Fig. 2. MSP58 interacts with the NH2-terminal of Daxx. A, top panel, schematic drawing of the full-length Daxx and its derivatives used in the yeast two-hybrid assay for their ability to interact with full-length MSP58. Numbers correspond with amino acid residues. Bottom panel, yeast strain L40 was co-transformed with the indicated combinations of Daxx-derived baits and Gal-AD prey containing full-length MSP58. The relative strength of protein interactions was determined by liquid β-galactosidase assay. β-Galactosidase activity was measured as described in Fig. 1B; the full-length Daxx and various Daxx deletion mutants used in the GST pull-down assay are shown schematically (left panel). Numbers indicate the amino acid position. The GST-MSP58 fusion protein and GST control protein were purified as instructed by the manufacturer (Amersham Biosciences). Five microliters of in vitro translated [35S]methionine-labeled Daxx and various deletion mutants were incubated with the GST-MSP58, or GST bound to glutathione-Sepharose beads in a pull-down assay as described under “Experimental Procedures.” The samples were washed and analyzed by SDS-PAGE and autoradiography. Input lane represents 20% amount of [35S]Daxx fragments subjected to the GST pull-down assay. Coomassie Blue staining of GST and GST-MSP58 used in GST pull-down assay is shown in bottom panel.

MSP58 Facilitates Nucleolar Sequestration of Daxx

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25450 MSP58 Facilitates Nucleolar Sequestration of Daxx

FIG. 3. MSP58 binds to Daxx in vivo. A, COS-1 cells were transfected with the indicated expression vectors. For protein expression, 20 μg of cell lysates were subjected to Western blot analysis with anti-FLAG or anti-HA antibody (bottom two panels). Immunoprecipitation (IP) was carried out from 500 μg of total cell extracts with the indicated antibody, and the precipitated proteins were analyzed by Western blotting (WB) as indicated. The antibodies used are indicated on the right side of the panels. The arrowhead points to the position of the co-precipitated MSP58, whereas the star indicates heavy chain of IgG. B, mapping of the MSP58 interaction domain of Daxx. COS-1 cells were co-transfected with MSP58 alone or with HA-tagged Daxx mutant fragments as indicated. The expression levels of FLAG-MSP58, HA-Daxx, and mutants were determined (bottom panel). Approximately 500 μg of total cell extracts were subjected to IP with anti-FLAG antibody followed by WB with anti-HA antibody (top panel). FL, full-length.

two-hybrid assays, the COOH terminus-deleted Daxx-(1–625) or Daxx-(1–501) bound to GST-MSP58, whereas Daxx-(501–740) failed to do so. To locate the domains in the Daxx that interact with MSP58, Daxx-(1–501) fragment was further engineered into two separate fragments, Daxx-(1–250) and Daxx-(250–501), and assayed by the GST pull-down assay. Clearly, both Daxx-(1–250) and Daxx-(250–501) can specifically interact with MSP58 to the extent compared with that of Daxx-(1–501). As a negative control, no interaction was observed between the COOH-terminal fragment Daxx-(625–740) and MSP58 despite that COOH-terminal region of Daxx (amino acid residues 625–740) was previously identified as a binding domain for other interacting proteins including Fas, PML, Pax3, Ubc9, and SUMO-1. Taken together, the results from our in vitro studies further confirm our observation made in the yeast two-hybrid experiments. In addition, the NH2-terminal region of Daxx is sufficient and indispensable for its interaction with MSP58.

Interaction of MSP58 with Daxx in Vivo—To establish whether MSP58 interacts with Daxx in mammalian cells, COS-1 cells were co-transfected with expression constructs encoding FLAG-MSP58 and HA-Daxx. Forty-eight hours after transfection, cell lysates were subjected to immunoprecipitation assays with anti-FLAG antibody followed by Western blot analysis with anti-HA antibody. As shown in Fig. 3A, Daxx was detected in the immunoprecipitated complexes of MSP58 (top panel, lane 4). This interaction was also validated in a reciprocal co-immunoprecipitation assay (second panel, lane 4). To further confirm that the subdomain of Daxx is involved in MSP58 binding, we also used various engineered Daxx expression constructs harboring Daxx-(1–625), Daxx-(1–501), and Daxx-(501–740) fragments, respectively, in co-immunoprecipitation experiments with MSP58. The protein expression levels of full-length Daxx deletion mutants Daxx-(1–625) and Daxx-(1–501) were comparable. However, the Daxx-(501–740) fragment was detected as a broadly migrating species on immunoblots. At present, it is not clear whether the high molecular weight form of Daxx-(501–740) reflects further posttranslational modification. This putative modification of Daxx-(501–740) was reproducibly observed in the lysates of transfected 293T or HeLa cells (data not shown). Notably, the deletion of the NH2-terminal region of Daxx resulted in a loss of its association with MSP58 as demonstrated by a lack of detection of the HA-Daxx-(501–740) protein in the immunoprecipitates of MSP58 (Fig. 3B, lane 6). Conversely, the COOH-terminal region deletion mutants Daxx-(1–625) and Daxx-(1–501), similar to wild-type Daxx, retained its interaction with MSP58 (Fig. 3B, lane 3–5). In consistent with our in vitro binding studies (Fig. 2), our findings suggest that the Daxx and MSP58 form a complex in cell, and the NH2-terminal region of Daxx is necessary and sufficient for its specific interaction with MSP58.

Cell Type-specific Effect of MSP58 on Daxx-mediated Transcriptional Repression—Because MSP58 was identified as a Daxx-interacting protein and Daxx is known to possess strong transcriptional repression activity, we decided to investigate the role of MSP58 in the regulation of transcriptional repression activity of Daxx. To achieve this goal, Gal4-Daxx was co-transfected with the increasing amounts of full-length MSP58 into COS-1 and HeLa cells. The transcriptional activity of Daxx mediated repression. In addition, consistent with previous reports (10, 15), the expression of PML but not PML
SUMO inhibits Daxx-mediated transcriptional repression both in COS-1 and HeLa cells (data not shown). Because cell context and promoter organization may alter the effect of transcription factor activity, the effect of MSP58 on Daxx transcriptional potential was re-examined in human 293 cells. Moreover, it was previously reported that the COOH-terminal region (amino acids 501–740) of Daxx is sufficient to repress the basal activity of a heterologous thymidine kinase promoter (8) but is unable to interact with MSP58 (Figs. 2 and 3). Hence, we also tested the effect of MSP58 on the trans-repression activity elicited by Gal4-Daxx-(501–740) fusions. As demonstrated in COS-1 cells, MSP58 in 293 cells also attenuated the repression activity of Gal4-Daxx in a dose-dependent manner, whereas MSP58 had little effect on reporter expression repressed by Gal4-Daxx-(501–740) fusions. The expression levels, estimated by Western analysis, of MSP58 and various Gal4-Daxx fusion proteins in 293 cells were comparable to those observed in COS-1 and HeLa cells (data not shown). Therefore, the experiments further support the notion that (i) the NH2-terminal region of Daxx interacts with MSP58 in vitro and in vivo, and (ii) this interaction modulates Daxx trans-repression ability in a cell context-specific manner.

MSP58 Recruits Daxx to the Nucleolus—To elucidate the molecular mechanism underlying MSP58-mediated modulation of Daxx trans-repression potential, immunofluorescence microscopic analyses were performed. Because previous studies have shown that MSP58 was localized in microspherules in the nucleolus and that the overexpression of MSP58 protein in COS-7 cells resulted in marked enlargement of the nucleolus (19), we then examined whether MSP58 could alter the subcel-

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**FIG. 4. Inhibition of Daxx-mediated transcriptional repression by MSP58.** A, COS-1 or HeLa cells of 6-well plates were transiently transfected with 500 ng of the Gal4-DBD or Gal4-DBD-DaxxFL mammalian expression vector in the absence or presence of the indicated amounts of MSP58 expression vector together with a Gal4-dependent luciferase reporter and pCMV-β-galactosidase. The total amount of the transfected DNA was adjusted to be the same as with pcDNA3. Forty-eight hours after transfection, the activities of luciferase and β-galactosidase were determined as described under “Experimental Procedures.” Luciferase activity from each transfected cell was normalized with the β-galactosidase activity and presented as the percentage of luciferase activity in the Gal4-DBD only. Errors bars represent the mean ± S.D. from three independent determinations. B, COS-1 or 293 cells were transiently transfected with 500 ng of the Gal4-DBD, Gal4-DBD-DaxxFL, or Gal4-DBD-Daxx-(501–740) mammalian expression vectors in the absence or presence of the indicated amounts expression vector MSP58 together with a Gal4-dependent luciferase reporter and pCMV-β-galactosidase. Luciferase activity was measured, and the data were presented as described above. The bottom panels show immunoblots with anti-Gal4-DBD (WB: a Gal DBD) or anti-FLAG (WB: a Flag) antibody to indicate the protein expression levels of the Gal4-DBD, Gal4-DBD-DaxxFL, Gal4-DBD-Daxx-(501–740), and FLAG-MSP58 in an aliquot of the cell lysates of COS-1 cells, each condition corresponding to the histograms.
MSP58 Facilitates Nucleolar Sequestration of Daxx

Fig. 5. MSP58 recruits Daxx to the nucleolus in a cell type-specific manner. A, COS-1 cells were transfected with plasmid constructs expressing either HA-Daxx alone (panels a–c), FLAG-MSP58 alone (panels d–f), HA-Daxx and FLAG-MSP58 (panels g–l), HA-Daxx-(501–740) and FLAG-MSP58 (panels m–o), or PML and HA-Daxx (panels p–r) as indicated in the lower part of the image. The antibodies used were the M2 monoclonal antibody against FLAG-tagged MSP58, the PG-M3 monoclonal antibody against PML, and a rabbit polyclonal anti-HA antibody against the HA-tagged Daxx. The red signal (HA-Daxx or HA-Daxx-(501–740)) was obtained with a Texas Red-conjugated secondary antibody. The green signal (FLAG-MSP58 or PML) was obtained with a fluorescein-conjugated secondary antibody. 4',6-Diamidino-2-phenylindole (DAPI) staining revealed the position of the nucleus (panels b, e, and r). The same cells were viewed with phase-contrast microscope (panels c, f, i, l, and o). The bold arrows in panels f, i, l, and o point to the nucleolus of transfected cells. The arrowheads point to the nucleolus of the non-transfected cells. HA-Daxx was localized to the nucleus (panels a–c), whereas FLAG-MSP58 showed accumulation in the nucleolus (panels d–f). Panels g–l show FLAG-MSP58 and HA-Daxx co-localization in the nucleolus. Panels m–o show that FLAG-MSP58 cannot recruit the HA-Daxx-(501–740) mutant lacking the MSP58-interacting domain to the nucleolus. Panels p–r show PML and HA-Daxx co-localization at the PODs. B, HeLa cells were transfected with plasmid constructs as indicated in the image. The immunofluorescence staining was carried out as described above. Panels a & b and c & d show diffuse nuclear staining of HA-Daxx and FLAG-MSP58, respectively. Panels e–h show FLAG-MSP58 and HA-Daxx co-localization at the nucleus. Panels i–j show PML and HA-Daxx co-localization at the PODs. C, 293 cells were transfected with the plasmid construct FLAG-MSP58 (panels c and d). The immunofluorescence staining was carried out as described above. Panel a shows diffuse nuclear staining of endogenous Daxx by anti-Daxx antibody. Panel c indicates that endogenous Daxx is recruited to nucleoli upon the expression of MSP58.
lular localization of Daxx in the nucleus. In these studies, COS-1 and HeLa cells were transiently transfected with a combination of FLAG-MSP58 and HA-Daxx and subsequently stained with the mouse anti-FLAG and/or rabbit anti-Daxx antibodies followed by immunofluorescence analysis. When HA-Daxx was overexpressed alone, a fairly diffused and evenly distributed staining pattern was observed in the nucleus both in COS-1 and HeLa cells (Fig. 5A and B, panels a and b). Consistent with a previous report (19), MSP58 was predominantly localized in the nucleolus in COS-1 cells (Fig. 5A, panels d, g, j, and m) and was rendered an increase in the nucleolar volume compared with that in the non-transfected cells (Fig. 5A, panels f, i, and o, arrow versus arrowhead), albeit the level of enlargement in our studies was repeatedly lower than that reported previously (19). The discrepancy may be attributed to different cell subtypes used in the studies. By contrast, MSP58 was indiscriminately found in the nucleus with a diffused pattern in HeLa cells (Fig. 5B, panel c) when MSP58 was expressed alone. Unlike in COS-1 cells, the sizes of nucleolus were grossly similar among the transfected and non-transfected HeLa cells (Fig. 5B, panel d). Interestingly, the co-transfection of MSP58 drastically altered the distribution of Daxx in COS-1 cells, because nearly all of the Daxx protein was recruited to the nucleolus, suggesting that MSP58 associates with Daxx and subsequently translocates the complex to the nucleolus (Fig. 5A, panels g–l). The effect of MSP58-induced Daxx nucleolar accumulation is comparable to the sequestration of Daxx to the PODs by PML caused enlarged PODs (Fig. 5A, panels p–r). In contrast, the co-transfection of MSP58 did not recruit a Daxx mutant Daxx-(501–740) to the nucleolus (Fig. 5A, m–o), correlating Daxx-MSP58 interaction with the process of Daxx recruitment to the nucleolus. To ensure Daxx-MSP58 interaction, the Daxx-(501–740) fragment containing the PML-binding domain was capable of being recruited to PODs by overexpressed PML (data not shown). Notably, the diffused nuclear distribution of HA-Daxx was not altered upon the co-expression of MSP58 in HeLa cells, despite that the Daxx and MSP58 were co-localized within the nucleus (Fig. 5B, panels e–h). By contrast, PML was capable of recruiting Daxx to PODs as demonstrated by a dramatic increase in the size of the PODs (Fig. 5B, panels i and j) in HeLa cells. Taken together, we conclude that the ability of MSP58 to derepress Daxx trans-repression resides within the potential by MSP58 to recruit Daxx to the nucleolus. 

MSP58 Inhibits Daxx-mediated Repression of Glucocorticoid Receptor Transcriptional Activity through Sequestration of Daxx in the Nucleoli—we have recently revealed that Daxx interacts with GR and suppresses GR-mediated transactiva-

![Graph](http://www.jbc.org/)

**Fig. 6.** MSP58 inhibits Daxx-mediated repression of GR transcriptional activity. A, 293 cells cultured on 6-well plates were transiently transfected with mammary tumor virus long terminal repeat-luciferase reporter, β-galactosidase, and plasmid constructs expressing GR, HA-Daxx, and FLAG-MSP58 as indicated. The total amount of the plasmid transfected was kept constant by adding empty pFlag-CMV-2 as needed. The cells were treated with 10 nM Dex (w/ Dex) or vehicle (w/o Dex) 18 h after transfection. Luciferase activity was determined as described under “Experimental Procedures.” The reporter gene activities were reported as relative light units and were represented as the mean ± S.D. B, 293 and HeLa cells were transfected with the expression constructs as indicated. The cells were subjected to Dex treatment followed by the analysis of luciferase reporter gene activity as described above.
To further substantiate that MSP58 can antagonize the action of Daxx in transcriptional repression, we analyzed the effect of MSP58 on GR-mediated transactivation. 293 cells were transiently transfected with a combination of expression vectors encoding GR, Daxx, and MSP58 together with the reporter construct mammary tumor virus long terminal repeat-luciferase reporter, which contains the GR recognition site. As shown in Fig. 6A, the overexpression of Daxx resulted in a suppression of Dex-induced GR transactivation. The co-expression of MSP58 was able to relieve the repressive effect of Daxx on GR transcriptional activity in a dose-dependent manner. Furthermore, when MSP58 was expressed along with GR in the absence of Daxx overexpression, an increment of the reporter on GR-mediated activation was observed in 293 cells (Fig. 6B, top panel). This observation could be attributable to the ability of MSP58 to relieve the repressive effect of endogenous Daxx through sequestration to the nucleoli in 293 cells. As shown in Fig. 5C, endogenous Daxx in 293 cells could be sequestered to nucleoli upon the expression of MSP58. For a specificity control of MSP58 in regulating GR activity, we analyzed the transactivation activity of GR in HeLa cells along with MSP58 expression. As expected, the overexpression of MSP58 in HeLa cells failed to potentiate the GR-mediated transactivation (Fig. 6B, bottom panel), suggesting that the potential of MSP58 in recruiting Daxx to nucleoli determines the specificity of MSP58 in regulating GR transcriptional activity. In summary, the results from transient co-transfection assays and immunofluorescence studies unequivocally indicate that MSP58 relieves the Daxx-repressive effect by diverting Daxx from its natural targeted genes in the nucleoplasm to the nucleolus.

**DISCUSSION**

In this study, we have identified MSP58 as a Daxx-interacting protein and demonstrated that MSP58 regulates the transcriptional repressor activity of Daxx through the alteration of Daxx subnuclear localization. Human MSP58 is a nucleolar protein that was identified initially to interact with the proliferation-related nucleolar protein p120 (19). We provided biochemical evidence that MSP58 interacts with Daxx in yeast in vitro and in mammalian cells. Moreover, the co-expression of MSP58 relieves the transcriptional repression by Daxx, correlating with the recruitment of Daxx from the nucleoplasm to the nucleolus. Thus, these findings raise an interesting possibility that nucleolus may play an important role in modulating Daxx transcription repressor activity.

Our studies further suggest an important regulatory role for the NHL-terminal region of Daxx. Daxx encodes a protein of 740 amino acids and contains a Ser-Pro-Thr-rich COOH terminus and a region rich in acidic amino acids commonly found in many transcriptional regulators (1, 22). Interestingly, the COOH-terminal region of Daxx corresponding to residues 625–740 has been reported to be necessary and sufficient for binding to Fas and required for enhancing Fas-induced apoptosis (1). The same region also displayed the ability to repress transactivation and for efficient targeting to PODs (8). It is curious that essentially the same region of Daxx has been found to interact with a wide variety of molecules including PML, CENP-C, 2 Lin, D.-Y., Hung, C.-C., and Shih, H.-M., manuscript in preparation.

![FIG. 7. A model for the compartmental modulation of Daxx-elicited transcriptional repression.](http://www.jbc.org/)

Daxx exerts its transcriptional repression effect in the nucleoplasm. This repressive effect can be relieved by protein-protein interactions, which shuttle Daxx to different subcellular compartments.
Pax3, ETS1, Ubc9, and SUMO-1 in yeast two-hybrid system (10, 12, 13, 16, 23), suggesting that the COOH-terminal region of Daxx functions as a docking domain for protein-protein interactions. Currently, it is not clear how this domain provides a binding surface for these proteins with distinctive functions. Despite these findings, there is so far a lack of unifying theme on the putative role of Daxx in governing cell behavior. Hence, it is reasonable to presume that protein modifications and/or direct or indirect protein-protein interactions are essential for proper Daxx function in different cellular contexts. As shown in Fig. 3B, the expression of HA-Daxx-(501–740) as in COS-1 cells was detected as a broad migrating band on immunoblots as reported previously (16), suggesting that the COOH-terminal of Daxx may be a target for posttranslational modification in vivo. This region at least in part is demonstrated to be phosphorylated by multiple signaling events (12).

In contrast to the studies of Daxx COOH-terminal region, the function of Daxx NH2-terminal region remains largely unknown. To our knowledge, this is the first report of which the NH2-terminal region of Daxx corresponding to amino acids 1–501 is involved in its interaction with MSP58 and subsequent translocation to the nucleolus. This region is predicted to harbor two coiled coil-like structures (amino acid residues 180–212 and 356–388) (16) and two paired amphipathic helices (PAHs) (PAH1, amino acid residues 64–76 and 96–108, and PAH2, amino acid residues 192–205 and 230–242) (12) that share conserved amino acids with four other PAHs reportedly present in the yeast transcriptional co-repressor Sin3 and its mammalian homologue Sin3a (24, 25). Both the coiled coil domain and the PAH domains have been proposed to function as protein-protein interaction domains (26). Our finding that Daxx-(1–250) or Daxx-(250–501) is capable of interacting with MSP58 raises a possibility that the Daxx-MSP58 interaction may be through coiled coil domains and/or PAH1 and PAH2 domains. Further investigation is required to address this possibility.

It is highly probable that the physiological role of Daxx would be more complex than what we currently know. A role for Daxx in transcriptional repression identified Daxx initially as interacting with Pax3 (12), a member of the paired class homeodomain family of transcription factors. Daxx was shown to repress the transcriptional activity of Pax3 by 80% (12). Daxx has also been reported to repress the basal activity by a heterologous thymidine kinase promoter through Gal4-DBD-Daxx fusion (8) as well as the transcriptional activity of ETS1 (13). In this study, we also demonstrated that Daxx is capable of suppressing Dex-induced transactivation of GR. Although the notion that Daxx plays a role in transcriptional repression in the nucleus is well accepted, the exact mechanism by which it can be controlled is yet to be clear. One intriguing possibility that has recently been put forward is that Daxx translocates to the PODs (PML oncogenic domains) also referred to as nuclear domain 10 by the interaction with PML. The overexpression of PML reverses the transcriptional repression mediated by Daxx (10). Interestingly, such a derepression correlates well with the sequestration of Daxx to the PODs by sumoylated PML. In this report, we have shown that the transcriptional repression and the localization of Daxx can be modulated by the nucleolus-associated MSP58 protein. In particular, our results suggest that specific cell context could alter the effect of MSP58 protein on Daxx function and localization. Moreover, MSP58 overexpression in COS-1 cells or 293 cells leads to the irregular enlargement of their nucleoli. Conversely, transfected MSP58 indiscriminately distributed through the nuclei of HeLa cells, and the size of nucleolar pattern was essentially unaltered. The mechanism underlying this observed discrepancy on MSP58 subcellular localization among the different cell types is currently not known. One possibility is that a putative factor(s) required for targeting MSP58 to nucleolus is present in COS-1 and 293 cells but not in HeLa cells. Alternatively, a putative factor(s) with ability to prevent MSP58 from accumulating in nucleolus is present in HeLa cells. Further study is definitely required to explore the molecular mechanism for this phenomenon.

Protein shuttling between different subcellular compartments may play an important regulatory step for Daxx functions, and Daxx potentially performs multiple functions depending on its cellular milieu. The role of Daxx as an adaptor linking ASK1 to Paf depending on its cellular localization and cell type has recently been re-investigated (5). Daxx can be trapped in the cytoplasm via ASK1 association, which correlates with the findings that the repressive effect of Gal4-Daxx was abolished by the co-expression of ASK1 (5). In addition, Daxx can also be relocated to PODs via its interaction with PML. The POD compartment has been proposed to enhance gene expression through the recruitment of activators (27, 28). Alternatively, PODs may participate to the transcriptional activation of specific target genes by tethering co-repressors like Daxx from the diffuse nuclear fraction where transcription takes place. Indeed, the fact that Daxx concentrates at condensed chromatin in PML–/– cells (9) supports the idea that Daxx is likely to exert its repressive properties outside of the PODs. Our findings that MSP58 such as PML modulates Daxx transcriptional repression activity via recruitment to nucleolus lead to a hypothesis that the nucleolus and PODs may function as distinctive reservoir in regulating the shuttling of Daxx among different subcellular compartmentalizations upon the specific protein-protein interactions as illustrated in Fig. 7. The dynamic feature of PODs and nucleolus on modulating Daxx function suggests that both subnuclear structures may serve as a flexible protein-based scaffold to regulate protein functions. It can also be speculated that PML or MSP58 sequesters Daxx to PODs or nucleoli, respectively, where Daxx can be stored in an inactive state or subjected to the protein degradation pathway, thereby altering the Daxx-mediated transcriptional regulation of target genes. Additional studies are required to address the underlying mechanism and functional significance of the sequestration of Daxx protein to these distinct nuclear substructures.

Very little is known regarding the function of MSP58. Besides its interaction with a proliferation-associated protein p120, a quail homologue of MSP58, TOJ3, has recently been cloned, and its expression has been up-regulated by v-Jun in a quail cell line (29). The overexpression of TOJ3 in cells can lead to an anchorage-independent cell growth in soft agar, which is similar to the cell transformation induced by v-Jun (29). Currently, the molecular mechanism of MSP58-associated cellular transformation is unclear. Whether that Daxx translocation to nucleolus facilitates the process of MSP58-induced cell transformation would be an interesting question to be explored. In conclusion, our findings reveal a novel function of MSP58 and nucleolus compartment in the regulation of Daxx-mediated transcriptional repression.

Acknowledgments—We thank Dr. David K. Ann for critical comments on the manuscript. We also thank Drs. David K. Ann, Chawnschang Chang, and Ronald M. Evans for plasmid constructs.

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MSP58 Facilitates Nucleolar Sequestration of Daxx
