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Sumoylation of Daxx Regulates IFN-Induced Growth Suppression of B Lymphocytes and the Hormone Receptor-Mediated Transactivation

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Daxx has been shown to play an essential role in type I IFN-mediated suppression of B cell development and apoptosis. Recently, we demonstrated that Tyk2 is directly involved in IFN signaling for the induction and translocation of Daxx, which may result in growth arrest and/or apoptosis of B lymphocyte progenitors. To clarify the molecular mechanisms of how Daxx acts on growth suppression of B lymphocytes, we examined functions of a sumoylation-defective Daxx KA mutant (Daxx K630/631A), which substituted Lys 630 and Lys 631 to Ala. Importantly, Daxx KA localized in the cytoplasm, whereas wild-type Daxx localized in the nucleus. Murine pro-B cell line Ba/F3 expressing Daxx KA revealed a resistance to the IFN-induced growth suppression. It is noteworthy that treatment with an exportin inhibitor, leptomycin B, resulted in nuclear localization of Daxx KA and recovery of the IFN-induced growth suppression in Ba/F3 cells. Moreover, Daxx KA decreased the binding potential to promyelocytic leukemia protein (PML), and overexpression of PML recruited Daxx KA into PML oncopgenic domains. Notably, a Daxx-small ubiquitin-related modifier fusion protein exhibited increased nuclear localization and ability to suppress cell growth in Ba/F3 cells. These results demonstrate that the IFN-induced growth suppression of B lymphocytes requires nuclear localization of Daxx through its sumoylation and proper interactions with PML. The Journal of Immunology, 2006, 177: 1160–1170.

The type I IFNs mediate not only potent antiviral and antiproliferative activities on target cells but also the induction of MHC class I molecules in T cells and NO production by macrophages (1). Treatment of cells with IFNs induces the activation of Jak-STAT molecules, including Jak1 and Tyk2 as well as STAT1 and STAT2 (2). Previous experiments using knockout mice have revealed that the Jak-STAT signals are major pathways for the IFN-mediated functions (3–8).

The type I IFN is known to be a potent inhibitor of IL-7-dependent growth of early B cell lineage progenitors, effectively aborting further B cell lineage differentiation at the pro-B cell stage (9). Although many type I IFN-induced responses are aborted in STAT1-deficient mice (3, 5), the inhibition of IL-7-dependent B lymphopoiesis by the type I IFNs is unaffected by disruption of STAT1 (10). Our experiments using Tyk2-deficient mice revealed that Tyk2 is essential for the type I IFN-induced inhibition of colony formation of B lymphocyte progenitors in response to IL-7 as well as the up-regulation and nuclear translocation of Daxx (11). Because Daxx plays crucial roles in the type I IFN-induced growth suppression of B lymphocyte progenitors (12), it is very informative to identify molecular mechanisms of Daxx involved in the growth arrest and/or apoptosis in early B cell development.

Daxx was first identified as a Fas-binding protein by yeast two-hybrid screening and was known as a proapoptotic protein that can enhance Fas-mediated apoptosis through JNK activation (13). However, disruption of Daxx in mice increased apoptosis during the embryonic development, suggesting that Daxx acts as an antiapoptotic protein in the embryo (14). Because of the diverse effects of Daxx between in vitro and in vivo experimental systems, roles of Daxx in apoptotic signals still remain unclear. Although the interaction between Daxx and Fas indicated the importance of Daxx in cytoplasm, nuclear localization of Daxx was observed in various cell lines and the interactions of Daxx with several nuclear proteins, such as the centromeric protein CENP-C, DNA methyltransferase 1, Pax-3, Pax-5, ETS1, Ubc9, small ubiquitin-related modifier 1 (SUMO-1), and promyelocytic leukemia protein (PML) were reported (14–21). Thus, Daxx is likely to play alternative roles in shuttling between nucleus and cytoplasm. Recent studies also demonstrated that Daxx might function as a transcriptional coregulator. Daxx has been shown to possess transcriptional repression activity by inhibiting several transcription factors such as Pax3, ETS1, and glucocorticoid receptor (GR) through direct protein–protein interactions (16, 18, 22). Daxx also is shown to act as a transcriptional coactivator or corepressor of Pax5 in different cell types (17). Although the exact mechanism accounting for these observations is still unclear, the recruitment of nuclear factors possessing either histone acetyltransferase or histone deacetylase activity by Daxx to modulate transcriptional activity was proposed (17, 23). In addition, the transcriptional repression effect of Daxx could be modulated by subnuclear compartmentalization...
through protein–protein interactions (24–26). Furthermore, PML has been shown to relieve the transrepression effect of Daxx on Pax3 or GR transcriptional activity through sequestering Daxx into the PML oncosuppressor domains (PODs) (22, 27).

There are two major modification signals for Daxx, including the phosphorylation and the sumoylation of the Daxx protein (26, 28, 29). A Ser 669 residue of Daxx (identical with human Daxx Ser 668) is phosphorylated by HIPK1, and a mutation of Daxx Ser 669 to Ala increased Daxx-mediated transrepression (26). Thus, the phosphorylation of Daxx diminishes Daxx transcriptional repression of specific promoters. It also was reported that HIPK2 regulates Daxx phosphorylation and that HIPK2 and Daxx cooperatively activate the JNK signaling pathway and regulates TGF-β-induced apoptosis in p53-deficient hepatoma cells (29). Another posttranscriptional modification is sumoylation of Daxx. Substitution of Lys 630 and Lys 631 to Ala (K630/631A; KA) abrogated the modification of Daxx by SUMO-1 and Ubc9 E2-conjugating enzyme, which conjugates SUMO-1 but not ubiquitin (28). Recently, overexpression of a Ubc9 dominant-negative mutant, Ubc9-DN also has been shown to induce accumulation of cytoplasmic Daxx (30). However, the precise biological functions and molecular mechanisms of Daxx modification remain still unclear.

In this study, to clarify roles of Daxx sumoylation, we used a sumoylation-defective mutant, Daxx K630/631A (Daxx KA), whose Lys 630 and Lys 631 were substituted to Ala. Importantly, sumoylation modification of Daxx regulates its nuclear anchoring via the association with PML, of which impairment leads to the failure of IFN-induced growth suppression of B lymphocytes and of the inhibition of GR-mediated transactivation. Our results indicate a novel physiological role of Daxx sumoylation in IFN-mediated signaling and may suggest a novel clue for new anti-cancer therapeutic approaches.

Materials and Methods

Reagents and Abs

Recombinant mouse IFN-α was purchased from PBL Biomedical Laboratories. Recombinant mouse IFN-γ/limitin was described previously (31). Recombinant human IFN-α and leptotycin B (LMB) were provided by Sumitomo Pharmaceuticals and M. Yoshida (Chemical Genetics Laboratory, RIKEN, Wako, Japan) (32), respectively. Dexamethasone (Dex) was purchased from Wako Pure Chemical. Expression vectors for FLAG-tagged Daxx, murine mammary tumor virus-luciferase (MMTV-LUC), FLAG-tagged Ubc9, and SUMO1 were described previously (33, 34). Expression vectors for STAT1 and PML as well as GR were provided by J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN), I. Matsumura (Osaka University Medical School, Osaka, Japan), and Dr. F. Saatcioglu (University of Oslo, Oslo, Norway). Epitope-tagged Daxx K630/631A mutants and FLAG-tagged Ubc9 C93S mutant were generated by PCR methods and sequenced (primer sequences are available upon request) (33, 34). A Daxx–SUMO fusion construct, which tagged Daxx with SUMO-1 at the C terminus, also was generated by PCR methods (primer sequences are available upon request). The construct was sequenced to confirm the reading frame. Anti-Daxx (M-112), anti-PML (H-238), anti-STAT1 (E-23), and anti-CRM1 (H-300) Abs and anti-c-Myc mAb (9E10) were obtained from Santa Cruz Biotechnology. Anti-FLAG mAb (M2) was purchased from Sigma-Aldrich, anti-SUMO1 Ab from Boston Bioschim, and anti-actin mAb from Chemicon International.

Cell cultures

An IL-3-dependent murine pro-B cell line, Ba/F3, was maintained in RPMI 1640 medium supplemented with 10% FCS and 10% conditioned medium from WEHI-3B cells as a source of IL-3. A stable transformant expressing Daxx wild type (WT), Daxx KA, or Ubc9 was established as described previously (34). In brief, 20 μg of an expression vector for FLAG-Daxx WT, K630/631A, or FLAG-tagged Ubc9 in pDNA3 was transfected by electroporation, and stable clones were selected with G418 (0.5 mg/ml).

Transfection, nuclear fractionation, LUC, and sumoylation assays

MAMTV-LUC and GR, as well as the indicated plasmids, were transfected into 293T cells with the standard calcium precipitation protocol (34). Thirty-six hours after transfection, the cells were treated either with or without 100 nM Dex for 16 h. The cells were then lysed in 100 μl of the reporter lysis buffer (Promega) and assayed for LUC and β-galactosidase activities according to the manufacturer’s instructions. LUC activities were normalized to the β-galactosidase activities. A total of 5 × 10⁵ of Ba/F3 cells was transfected with GR, MAMTV-LUC, and pRL-TK-LUC together with Daxx WT or Daxx KA by electroporation (960 V, 330 V) with a Gene-pulsor II (Bio-Rad). Twelve hours after transfection, the cells were then stimulated either with or without Dex for 12 h. Cells were lysed in 50 μl of reporter lysis buffer for LUC assay. LUC assay was performed as described above. For the detection of sumoylation of Daxx in Ba/F3 cells, transient transfection of cells was performed using Nucleofector Solution V (Amaxa Biosystems). A total of 5 × 10⁵ of Ba/F3 cells was nucleofected with plasmids for Myc-Daxx WT or Myc-Daxx KA and/or FLAG-Ubc9 and SUMO-1 using 100 μl of Nucleofector Solution V according to the manufacturer’s instructions. Twelve hours after transfection, cells were lysed and immunoprecipitated with anti-Myc Ab.

Analysis of cell viability

The number of viable Ba/F3 cells with the indicated treatments was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay (Cell Counting Kit-8; Wako Pure Chemical). Ten microliters of WST-8 solution was added into each well, and the cells were incubated for 1 h. The absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader (Bio-Rad). Cell viability was evaluated as the ratio of the absorbance of the sample to that of the control.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously (34). Briefly, cells were harvested and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, containing 1% Nonidet P-40, 1 μM sodium orthovanadate, 1 μM PMSF, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on SDS-PAGE and transferred to Immobilon filter membranes. The filters were then immunoblotted with each Ab. Immunoreactive proteins were visualized using an ECL detection system (Amerham Biosciences).

Indirect immunofluorescence microscopy

To analyze the subcellular localization of Daxx proteins, FLAG-Daxx with or without the indicated vectors were transiently transfected into COS-7 or HeLa cells by Jet-PEI (PolyTransfection). Ba/F3 cells were spun down onto glass slides and fixed. LMB treatment (10 mg/ml) was performed prior to fixation (12 or 24 h). Immunofluorescence stainings were performed as described (34). The following primary Abs were used: mouse anti-FLAG M2 (1/1000), rabbit anti-Daxx (M112; 1/100), rabbit anti-PML (H-238; 1/100), rabbit anti-SUMO-1 (1/100), and rabbit anti-STAT1 (E-23; 1/100). Two secondary Abs were used: FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (Chemicon International). DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) (Wako Chemicals) staining. Confocal laser scanning microscopy was performed with an LSM510 microscope (Zeiss) with an Apochromat ×63/1.4 oil-immersion objective, using excitation wavelengths of 543 nm (rhodamine red) and 488 nm (FITC).

Statistical methods

The significance of differences between group means was determined by Student’s t test.

Results

Defective IFN-induced sumoylation in Daxx K630/631A mutant

To assess the molecular mechanisms involved in the IFN/Daxx-mediated signaling, we focused on posttranscriptional modification of Daxx using the Daxx KA mutant whose Lys 630 and Lys...
631 have been substituted to Ala (28). SUMO-1 is a small ubiquitin-related modifier that modifies various cellular proteins covalently, and the SUMO-1 modification demands a conjugation enzyme, Ubc9, which posttranslationally sumoylated Daxx (28). We first examined sumoylation of Daxx WT and Daxx KA in the presence of SUMO-1 and Ubc9 in 293T cells. Expression vectors for FLAG-tagged Daxx WT or Daxx KA together with or without plasmids for SUMO-1 and Ubc9 were transfected into 293T cells. As shown in Fig. 1A, Western blot analysis of the immunoprecipitate with anti-FLAG Ab revealed that sumoylation of Daxx WT, but not Daxx KA, was observed in the presence of SUMO-1 and Ubc9. We also tested whether IFN treatment has an effect on sumoylation status of Daxx. Time-course analysis of IFN-induced sumoylation demonstrated that the level of sumoylation of Daxx WT was highest at 2 h and sustained for 20 h (Fig. 1B). Importantly, IFN-treatment induced a strong enhancement of sumoylation of Daxx WT but not Daxx KA (Fig. 1A). We further analyzed the sumoylation status of several Daxx mutants. Daxx protein has been reported to exhibit at least two putative nuclear localization sequences from its amino acid sequence (15). One is around Lys 392 and Lys 393, and the other is around Lys 630 and Lys 631 corresponding to sumoylation sites described here. We substituted Lys 392 and Lys 393 to Ala and examined their sumoylation status. However, we could not detect any alteration of their sumoylation status, although the band of the sumoylation form was greatly reduced in the K630/631A mutant in the same experiments (data not shown). We also substituted a HIPK1 phosphorylation site, Ser 668 to Ala (26), and tested its sumoylation status. Similarly, no alteration of its sumoylation status was observed (data not shown). In our previous study, we showed that Tyk2 is essential for IFN-induced B lymphocyte growth inhibition, through the up-regulation and nuclear accumulation of Daxx, using Tyk2-deficient mice (11, 37). We also demonstrated that IFN-α inhibited the growth and up-regulated Daxx expression in an IL-3-dependent murine pro-B cell line, Ba/F3 (38). We then examined sumoylation of Daxx in Ba/F3 cells. Expression vectors for Myc-tagged Daxx WT or Daxx KA together with or without plasmids for SUMO-1 and Ubc9 were transiently transfected into Ba/F3 cells. As shown in Fig. 1C, Western analysis of the immunoprecipitate with anti-Myc Ab revealed that sumoylation of Daxx WT, but not Daxx KA, was observed in the presence of SUMO-1 and Ubc9. Therefore, it seems to be true that the K630/631A mutant has very little capacity to be sumoylated, although we cannot exclude the possibility that other Daxx sumoylation sites may exist.

Daxx K630/631A mutant suppresses Daxx-mediated GR repression and IFN-induced growth arrest in Ba/F3 cells

To first assess the functional relevance of sumoylation on Daxx, we examined the effect of sumoylation on transcriptional regulation by Daxx. Daxx is known to act as a transcriptional regulator (15–18, 21, 25, 27, 39). We previously demonstrated that overexpression of Daxx suppresses GR-mediated activation of the MMTV promoter in a human embryonic kidney carcinoma cell line, 293T cells (34). To examine the function of Daxx KA in the context of GR signaling pathway, we performed the transient transfection assay where the GR-mediated transcriptional responses were measured by MMTV-LUC (34). When 293T cells were transfected with MMTV-LUC together with an expression vector for GR and treated with Dax, LUC expression was increased by ~30- to 40-fold (Fig. 2A). Overexpression of Daxx WT suppressed the GR-mediated transactivation in a dose-dependent manner. However, overexpression of Daxx KA showed no suppressive effect on GR-mediated transactivation in 293T cells. To further confirm the enhanced effect of Daxx WT or Daxx KA on GR-mediated transcription in B lymphocytes, we transiently expressed GR and Daxx WT or Daxx KA together with the MMTV-LUC construct in Ba/F3 cells. As shown in Fig. 2B, Dex markedly augmented MMTV-LUC in Ba/F3 cells expressing GR. Overexpression of Daxx WT, but not Daxx KA, suppressed the GR-mediated transactivation in Ba/F3 cells. These results suggest that sumoylation of Daxx may be required for the transcriptional repressor activity in the GR-mediated transcription.
We next examined the effects of sumoylation of Daxx on IFN-induced growth suppression of B lymphocytes using Ba/F3 cells. We established stable transformants expressing Daxx WT or Daxx KA in Ba/F3 cells (Ba/F3 Daxx WT, Ba/F3 Daxx KA). Expression of the exogenous Daxx WT or Daxx KA protein was confirmed with Western blot analysis using anti-Daxx Ab (data not shown). The protein level of Daxx in transformants was ~5-fold higher than endogenous Daxx protein by densitometric intensity when compared using the results with anti-Daxx Ab (data not shown). As shown in Fig. 2, D and E, type I IFNs, IFN-α and IFN-γ limitin, induced growth arrest of Ba/F3 cells in a dose-dependent manner. Ectopic expression of Daxx WT enhanced IFN-induced reduction of cell viability. Importantly, ectopic expression of Daxx KA significantly inhibited IFN-induced reduction of cell viability. However, IL-3 deprivation-induced growth arrest or cell death occurred similarly in both types of transfectants (Fig. 2F), suggesting that Daxx KA specifically inhibits IFN-induced growth arrest in Ba/F3 cells.

Cytoplasmic localization of Daxx K630/631A mutant

We then attempted to examine whether IFN-treatment affects the subcellular localization of Daxx WT or Daxx KA. Expression vectors for FLAG-tagged Daxx WT or Daxx KA together with a plasmid for STAT1, was transfected into COS7 cells. Twenty-four hours after transfection, the cells were treated either with or without IFN, fixed and reacted with anti-FLAG and anti-STAT1 Ab, and visualized with FITC or rhodamine-conjugated secondary Ab. Importantly, the majority of Daxx KA localized in the cytoplasm, whereas Daxx WT localized in the nucleus (Fig. 3A). These subcellular localization patterns of Daxx WT or Daxx KA did not alter at both 30 min and 20 h after IFN stimulation. In contrast, STAT1 translocated from the cytoplasm into the nucleus within 30 min after IFN-stimulation. Twenty hours after treatment with IFN, STAT1 was again observed in the cytoplasm. We also observed similar cytoplasmic localization of Daxx KA in HeLa cells (data not shown). These results indicate that the sumoylation of Daxx on Lys 630 and Lys 631 is necessary for nuclear localization of Daxx and that Daxx does not regulate the subcellular localization of STAT1 after IFN-stimulation.

We next examined colocalization of Daxx WT or Daxx KA with endogenous PML, which localizes in PODs within the nucleus. As shown in Fig. 3B, Daxx WT colocalized with endogenous PML in PODs in COS7 cells. However, Daxx KA failed to colocalize with PML in PODs, because Daxx KA localized in the cytoplasm. Interestingly, overexpression of Daxx KA, but not Daxx WT, reduced the number of PODs within the nucleus by ~60%,
FIGURE 3. Cytoplasmic localization of Daxx K630/631A mutant. A, COS7 cells (6-well plate) were cotransfected with FLAG-tagged Daxx WT or Daxx KA (1 μg) together with STAT1 (1 μg). Twenty-four hours after transfection, the cells were treated with or without IFN-α (500 U/ml) as indicated, fixed and reacted with anti-FLAG M2 and anti-STAT1 Abs, and visualized with FITC- or rhodamine-conjugated secondary Abs. (Figure legend continues)
suggesting that the absence of Daxx in the nucleus may have some additional effects on the PODs formation in the nucleus. We also assessed colocalization of Daxx WT or Daxx KA with ectopically expressed SUMO-1. As shown in Fig. 3C, ectopically expressed SUMO-1, as well as Ube9 (data not shown), in part colocalized with Daxx WT, but not Daxx KA, in PODs. Similar to the above results, the number of PODs also decreased in the presence of Daxx KA. We also tested whether a Ube9 dominant-negative mutant (Ube9 C93S) has an effect on the subcellular localization of Daxx. Recently, overexpression of Ube9 C93S has been shown to induce accumulation of cytoplasmic Daxx (30). As shown in Fig. 3D, we could observe the similar effect of Ube9 C93S on the subcellular localization of Daxx. When Ube9 C93S, but not Ube9 WT, was expressed with Daxx, Daxx did not localize within the nucleus in ~60% of transfected cells, suggesting that the Ube9-mediated sumoylation is involved in nuclear localization of Daxx.

We also examined the subcellular localization of Daxx WT or KA in Ba/F3 cells. As shown in Fig. 4A, Daxx KA localized in the cytoplasm in Ba/F3 cells as did in COS7 and HeLa cells. However, Daxx WT in Ba/F3 cells localized to punctate structures in the nucleus while overexpression of Daxx WT in COS7 or HeLa cells localized in the nucleus with a diffuse pattern (Figs. 3, A and B, and 4A). Because endogenous Daxx has been known to localize in PODs as a dotted structure (20, 40, 41), these facts may suggest that the punctate feature of Daxx within nucleus may depend on protein contents and the protein–protein interaction within the nucleus. At the present time, we do not have any precise idea whether this difference pattern of Daxx depends on cell type or protein contents. We next tested colocalization of SUMO-1 with Daxx in Ba/F3 cells. As shown in Fig. 4B, after IFN-γ-treatment, endogenous SUMO-1 colocalized to punctate structures in the nucleus with Daxx WT, but not Daxx KA. Interestingly, cytoplasmic Daxx partly colocalized with endogenous SUMO-1 in the cytosol. These results demonstrate that Lys 630 and Lys 631 are required for nuclear localization of Daxx.

**Daxx K630/631A mutant fails to reside in the nucleus**

We next tested whether cytoplasmic localization of Daxx KA is due to the failure of nuclear import or nuclear retention of Daxx protein. To this purpose, we examined the effect of LMB, which inhibits nuclear export (NE) of Daxx, on cytoplasmic localization of Daxx KA. The cellular target responsible for the nuclear export of Daxx was identified as chromosomal region maintenance 1 (CRM1), which is a carrier protein for nuclear export and a receptor for the NE signal of Daxx (42). Binding of Daxx to CRM1 was inhibited by LMB, a specific inhibitor of CRM1-dependent NE (42). Expression vectors for FLAG-tagged Daxx WT or Daxx KA were transfected into COS7 cells. Twenty-four hours later, the cells were treated with LMB for 12 h. As shown in Fig. 5, A and B, LMB treatment resulted in nuclear localization of Daxx KA as well as Daxx WT. We also tested the binding potential of Daxx WT or Daxx KA to endogenous CRM1. As shown in Fig. 5C, Western blot analysis of the immunoprecipitate with anti-Myc Ab revealed that both Daxx WT and Daxx KA equally interact with
FIGURE 5. Nuclear accumulation of Daxx K630/631A restores IFN-induced suppression of Ba/F3 cell growth. A, COS7 cells (6-well plate) were transfected with plasmid expressing FLAG-tagged Daxx WT or Daxx KA (1 μg). Twenty-four hours after transfection, the cells were treated with or without LMB (10 ng/ml), fixed and reacted with anti-FLAG M2 Ab, and visualized with rhodamine-conjugated secondary Ab. The same slide was also stained with DAPI for the nuclei staining. B, Quantitative analysis of nuclear localization of Daxx KA. The number of cells showing rhodamine signal in the nucleus was calculated. The results shown are averages of three independent experiments, with SDs, in which 200 cells were counted. C, 293T cells (1 × 10^7) were transfected with plasmids to express Myc-tagged Daxx WT or Daxx KA (10 μg). Forty-eight hours after transfection, the cells were lysed and immunoprecipitated with anti-Myc Ab and immunoblotted with anti-CRM1 Ab (top panel) or anti-Myc Ab (middle panel). Total cell lysates (1%) were blotted with anti-CRM1 Ab (bottom panel). D, Ba/F3 Daxx KA cells were treated with or without LMB (0.3 ng/ml) for the indicated periods, and the cells were fixed and reacted with anti-FLAG M2 Ab, and visualized with rhodamine-conjugated secondary Ab. E, Ba/F3 pcDNA or Ba/F3 Daxx KA cells (1 × 10^7) were treated with the indicated concentrations of IFN-α with or without LMB (0.3 ng/ml) for 24 h, and the cell viability was determined by Cell Counting Kit-8.
endogenous CRM1. These results show that cytoplasmic localization Daxx KA is due to the failure of nuclear anchoring of Daxx KA, although Daxx KA properly interacts with CRM1.

**Nuclear accumulation of Daxx K630/631A restores IFN-induced suppression of Ba/F3 cell growth**

We further examined whether nuclear accumulation of Daxx KA by LMB has an effect on IFN-induced suppression of Ba/F3 cell growth. As shown in Fig. 5D, time course of LMB treatment showed the nuclear accumulation of Daxx KA in Ba/F3 cells, although LMB treatment did not induce Daxx expression in cells (Fig. 5E). Furthermore, LMB treatment for 24 h did not affect cell viability (data not shown). We then tested the effect of LMB treatment on IFN-induced suppression of cell growth. LMB-treated Ba/F3 Daxx KA cells, but not untreated cells, showed a marked IFN-induced reduction of cell viability (Fig. 5F). These data strongly suggest that IFN-induced suppression of Ba/F3 cell growth requires nuclear anchoring of Daxx protein, which is necessary for modification of SUMO-1.

**Impaired interaction of Daxx K630/631A with PML**

It has been demonstrated that the localization of Daxx into PODs is disrupted in PML-deficient cells, whereas ectopic expression of PML into PML-deficient cells causes relocalization of Daxx to PODs (20). Moreover, forced expression of Daxx induced apoptosis in PML+/+ cells, but not in PML−/− cells (41), indicating the involvement of PML in nuclear localization of Daxx. To assess this possibility, we examined the binding potential of Daxx WT or Daxx KA to PML in vivo. Expression vectors for PML together with Myc-tagged Daxx WT or Daxx KA, were transfected into 293T cells. As shown in Fig. 6A, a much larger amount of Daxx WT protein was contained than Daxx KA protein in the immunoprecipitates with anti-PML Ab, suggesting that Daxx KA has a binding potential to PML but not enough for anchoring itself in the nucleus. We then tested whether overexpression of PML overcomes defect of nuclear localization of Daxx. Notably, overexpression of PML together with Daxx KA resulted in relocation of Daxx KA into PODs similar to the cases of Daxx WT (Fig. 6B). These results indicate that the binding affinity of Daxx KA to PML is much lower than that of Daxx WT, which plays an essential role for nuclear localization, especially within PODs, of Daxx.

**Daxx-SUMO induces growth suppression and Ubc9 enhances IFN-induced growth suppression in Ba/F3 cells**

To more clarify the effect of sumoylation of Daxx on its subcellular localization, we used a Daxx-SUMO fusion protein, which tagged Daxx with SUMO-1 at the C terminus. It has been recently shown that SUMO-fusion proteins behave similarly to that of physiologically sumoylated proteins (43). As shown in Fig. 7, A and B, Daxx-SUMO fusion protein was expressed well and localized throughout the nucleus in Ba/F3 cells, although Daxx WT localized to punctate structures in the nucleus (Fig. 4A). We also examined the binding potential of Daxx-SUMO fusion protein to PML in vivo. Expression vectors for PML together with Myc-tagged Daxx WT, Daxx KA, or Daxx-SUMO were transfected into 293T cells. As shown in Fig. 7B, much larger amount of PML was contained in the immunoprecipitates with anti-Myc Ab, suggesting that Daxx-SUMO has much more binding potential to PML than Daxx WT. We finally tested whether Daxx-SUMO expression has an effect on Ba/F3 cell growth. As shown in Fig. 7C, a transient expression of Daxx-SUMO induced growth suppression in Ba/F3 cells. These data strongly suggest that sumoylation of Daxx is necessary for nuclear anchoring of Daxx via the interaction with PML and growth suppression in Ba/F3 cells. We further examined whether overexpression of Ubc9 has an effect on IFN-induced growth suppression in Ba/F3 cells. Because Ubc9 is an E2-conjugating enzyme required for sumoylation and also is implicated in regulating subcellular localization of Daxx as shown in Fig. 3D (30). We established stable transformants expressing Ubc9 in Ba/F3 cells (Ba/F3 Ubc9 #2 and Ba/F3 Ubc9 #4) whose exogenous

**FIGURE 6.** Overexpression of PML recruits Daxx K630/631A into PODs. A, A total of 1 × 10⁷ 293T cells was cotransfected with plasmids to express Myc-tagged Daxx WT or Daxx KA (10 μg) together with PML (10 μg). Forty-eight hours after transfection, the cells were lysed and immunoprecipitated with rabbit anti-PML Ab and immunoblotted with anti-Myc Ab (top panel) or anti-PML Ab (middle panel). Total cell lysates (1%) were blotted with anti-Myc Ab (bottom panel). B, HeLa cells (6-well plate) were cotransfected with Myc-tagged Daxx WT or Daxx KA (1 μg) together with PML (1 μg). Forty-eight hours after transfection, the cells were lysed, fixed and reacted with rabbit anti-PML and mouse anti-Myc Abs, and visualized with FITC- or rhodamine-conjugated secondary Abs. The same slide also was stained with DAPI for the nuclei staining.
Ubc9 expression was confirmed with Western blot analysis using anti-FLAG Ab (Fig. 7D; left panels). As shown in Fig. 7D, ectopic expression of Ubc9 enhanced IFN-induced growth suppression in Ba/F3 cells, suggesting that Ubc9 positively regulates IFN-induced growth suppression in Ba/F3 cells.

**Discussion**

In the present study, we identified a novel physiological role of sumoylation of Daxx by using a sumoylation-defective mutant (Daxx KA) whose Lys 630 and Lys 631 were substituted to Ala. Exogenously expressed Daxx KA proteins neither mediate signals to suppress cell growth in a Ba/F3 pro-B cell line nor inhibit GR-induced transcriptional activation in Ba/F3 and 293T cells. We also found the most important difference of their behaviors: Daxx KA localized in the cytoplasm, whereas Daxx WT localized in the nucleus. Notably, treatment of Daxx KA-expressing cells with LMB, a CRM-1/exportin inhibitor, enabled Daxx KA to accumulate in the nucleus as well as to mediate signals for growth suppression after IFN stimulation. These facts suggest that sumoylation of Daxx is essential for its residence in the nucleus, but not for...
its translocation from the cytoplasm into the nucleus, and that the accumulation of Daxx in the nucleus is likely to be required for IFN-induced growth suppression of B lymphocytes. Furthermore, we showed that Daxx KA bound to PML weaker than Daxx WT, and that overexpression of PML let Daxx KA reside in the nucleus. Thus, sumoylation of Daxx enhances the binding capacity to PML, which plays an essential role for nuclear localization of Daxx. We also showed that a Daxx-SUMO fusion protein exhibited increased nuclear localization and ability to suppress cell growth in Ba/F3 cells. Importantly, a dominant-negative form of a SUMO-1-conjugating enzyme, Ubc9, regulates the subcellular localization of Daxx and ectopic expression of Ubc9 enhances IFN-induced growth suppression in Ba/F3 cells. Taken together, our results strongly indicate that sumoylation modification of Daxx regulates its nuclear anchoring via the association with PML, of which impairment leads to the failure of IFN-induced growth suppression of B lymphocytes and of the inhibition of hormone receptor-mediated transactivation.

Posttranslational sumoylation of proteins is now recognized as an important modification system that can influence the function of a given protein. Sumoylation occurs by the covalent attachment of SUMO, a ubiquitin-related polypeptide, to lysine residues (44). Sumoylation also is known to regulate the functions of a target protein by changing the subcellular localization, protein–protein interactions, and/or stability (45). It has been reported that PML represents the major PODs structural constituent and depends on SUMO-1 for POD targeting (46). PML also has been shown to participate in several different apoptotic pathways (47). Overexpression of PML in various cell lines results in increased apoptosis (48). Conversely, PML-deficient mice are defective in induction of apoptosis by various stimuli. Therefore, the pro-apoptotic activity of PML could depend on its ability to heterodimerize with other factors involved in the regulation of apoptosis. Daxx also is known to localize in the nuclear body and interacts directly with PML (41). The pro-apoptotic activity of Daxx is abrogated in PML-deficient cells, where it acquires an aberrant microspeckled/patchy localization pattern in the nucleus (39). Therefore, the lower affinity of Daxx KA to PML affected Daxx-mediated functions on transcription regulation and growth suppression.

During the process of IFN-induced growth suppression of B lymphocytes, Daxx proteins are sumoylated and translocated into the nucleus. This study provides evidence that sumoylation of Daxx is essential for its interactions with PML, which may lead to nuclear anchoring of Daxx. We also have clarified that lack of sumoylation-dependent nuclear anchoring of Daxx results in a resistance to the IFN-induced growth suppression of B lymphocytes. We attempted to examine whether IFN treatment has an effect on sumoylation status of Daxx in Ba/F3 cells. Unfortunately, we could not detect a significant increase of sumoylation of Daxx in the immunoprecipitates in these cells because of the low detection sensitivity of anti-SUMO-1 Ab immunoblotting and the lower solubility of sumoylated proteins with Nonidet P-40. It is well documented that most sumoylated nuclear proteins, such as PML, translocate into the Nonidet P-40-insoluble fraction (46). Indeed, we could detect abundant sumoylated proteins in the lysates after boiling in the SDS sample buffer (data not shown). However, our results described in this study clearly indicate a novel physiologic role of sumoylation of Daxx in IFN-induced growth suppression and apoptosis. Recently, sumoylation of the orphan nuclear receptor, LRH-1, had an effect on its localization and transcriptional activity (49). Sumoylated LRH-1 was not associated with chromatin and its transcriptional activation of target genes. Desumoylation of LRH-1, which leads to the dissociation of LRH-1 from PODs, increased its transcriptional activity, suggesting that SUMO-PODs may function as dynamic molecular reservoirs, controlling the availability of certain transcription factors to active chromatin domains. Daxx may cooperate with PML and function as one of dynamic molecular reservoirs to controlling the IFN-induced gene activation or cell growth. Thus, manipulation of Daxx posttranscriptional modification is likely to provide a novel therapeutic strategy for B cell malignancies. In addition, regulation of sumoylation of Daxx may reduce adverse effects of IFNs, such as lymphomyelosuppression, because Daxx is a major mediator for IFN-induced growth suppression of megakaryocyte progenitors as well as B lymphocytes.

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Disclosures
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References
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