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SUMO Regulates the Cytoplasmonuclear Transport of its Target Protein Daxx

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Abstract It is known that Fas death domain-associated protein (Daxx) possesses both putative nuclear and cytoplasmic functions. However, the nuclear transport mechanism is largely unknown. This study examined the nuclear location signal (NLS) of Daxx and whether the nuclear transport of Daxx was mediated by small ubiquitin-related modifier (SUMO). Two NLS motifs of Daxx, leucine (L)-rich nuclear export signal (NES)-like motif (^{188}IXXLXXLLXL^{197}) and C-terminal lysine (K) rich NLS_2 (amino acids 627–634) motif, were identified and the K^{630} and K^{631} on the NLS_2 motif were characterized as the major sumoylation sites of Daxx by in vitro sumoylation analysis. Proteins of inactive SUMO (SUMO-Δ), a sumoylation-incompetent mutant, and Daxx NLS mutants (Daxx-NES^{mut} and Daxx $NLS_2{}^{mut}$) were dispersed in cytoplasm. The cytoplasmic dispersed Daxx mutants could be relocalized to nucleus by cotransfection with active SUMO, but not with inactive SUMO-Δ, demonstrating the role of SUMO on regulating the cytoplasmonuclear transport of Daxx. However, inactive SUMO-Δ could also be relocalized to nucleus during cotransfection with wild-type Daxx, suggesting that SUMO regulation of the cytoplasmonuclear transport of its target protein Daxx does not need covalent modification. This study shows that cytoplasmic SUMO has a biological role in enhancing the cytoplasmonuclear transport of its target protein Daxx and it may be done through the non-sumoylation interactions. J. Cell. Biochem. 98: 895–911, 2006.

Key words: Daxx; SUMO; cytoplasmonuclear transport; nuclear localization signals; non-sumoylation interactions

Human Fas death domain-associated protein (Daxx) is a 740-amino acids protein mainly localized in nucleus. It functioned as a transcriptional repressor when associated with chromatin in nucleus [Hollenbach et al., 1999, 2002]. Daxx-mediated repression of glucocorticoid receptor (GR) transcriptional activity was enhanced [Muromoto et al., 2004] by interacting with tumor susceptibility gene (*TSG*) 101. However, its transcription repression can be inhibited by the nuclear body-associated promyelocytic leukemia (PML) protein sequestering Daxx to the PML oncogenic domains (PODs) [Ishov et al., 1999; Li et al., 2000a; Lin et al., 2003], or by microspherule protein (MSP) 58 sequestering Daxx to the nucleolus [Lin and Shih, 2002]. Daxx's functions were terminated as its subnuclear localization changed [Muller et al., 1998; Ecsedy et al., 2003]. In cytoplasm Daxx was associated with cell surface and cytoplasmic molecules, including Fas [Yang et al., 1997] and transforming growth factor beta (TFG-β) [Perlman et al., 2001]. The increased cytoplasmic Daxx was more susceptible to apoptosis [Mo et al., 2004]. In addition, Daxx also interacts directly with apoptosis signal regulating kinase 1 (ASK1) and activates ASK1 in cytoplasm [Chang et al., 1998]. Daxx recruited to cytoplasm during overexpression of ASK1 has subsequently induced caspase-independent cell death [Charette et al., 2001].

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Daxx is a shuttle protein participating in biological functions on various subcellular localizations. The suggestion was that it contained both the nuclear localization signal (NLS) [Pluta et al., 1998] and the nuclear export signal (NES) [Song and Lee, 2004] for translocating into and exporting out of nucleus, respectively. The NES motif of Daxx has been identified on amino acid 565–575 residues by Song and Lee [2004], but the NLS motif of Daxx remains unclear. The lysine-rich consensus sequence proposed [Pluta et al., 1998] that Daxx contains two candidate NLS motifs, NLS_1 (391–395 a.a.) and NLS_2 (627–633 a.a.). The former, however, has been demonstrated to be non-functional by Daxx deletion mutant (1–625 a.a.) and site-direct mutagenesis [Torii et al., 1999]. Although the C-terminal Daxx (625–740 a.a.) localized in nucleus, its NLS_2 motif was regarded as non-functional by site-direct mutagenesis on both lysine 630 and 631 residues [Jang et al., 2002]. These two residues were regarded as the major sumoylation sites on Daxx by sumoylation assays in vivo by Jang et al. [2002].

Small ubiquitin-related modifier (SUMO) played a general role in regulating protein–protein interactions both in cytoplasm and in nucleus. In nucleus the treanscriptional activities of SUMO-1 modified transcription factors, including p53, c-jun, Sp-3, c-Myb, and c/EBP families were reduced [Gill, 2004]. It inhibits RAD51-mediated homologous recombination by interaction with RAD51 [Li et al., 2000b]. In cytoplasm SUMO-1 stabilized IκBα by blocking ubiquitination [Desterro et al., 1998] and modulated the partitioning of Ran-GAPase-activating protein (RanGAP1) between the cytosol and nuclear pore complex (NPC) [Matunis et al., 1996; Tatham et al., 2005; Reverter and Lima, 2005]. It represses ASK1 activation through physical interaction and not through covalent modification in cytoplasm [Lee et al., 2005]. Nevertheless, whether SUMO has a role in the cytoplasmonuclear transport is not completely understood.

It is reported in this paper that SUMO enhances the nuclear transport of Daxx. Three sumoylation sites of Daxx were determined by sumoylation assays in vitro and two NLS motifs of Daxx were identified. The cytoplasm mislocalized mutants of Daxx were used to analyze the nuclear transport function of SUMO and the non-sumoylation interactions between SUMO and Daxx are discussed.

MATERIALS AND METHODS

Plasmids

Plasmids encoding wild-type pAS2-SUMO, active pAS2-SUMO and inactive pAS2-SUMO-Δ deletion mutants were subcloned from SUMO cDNA sequences [Mannen et al., 1996] using appropriate oligonucleotides. The inactive SUMO-Δ were amplified by PCR from wild-type SUMO using forward primer of the wild-type and specific reverse primers to delete the amino acids beyond or including double glycines of the C-terminal of SUMO. The plasmids encoding wild-type pACT2-Daxx, pGEX-KG-p53 (377–393 a.a.), and pGEX-KG-PML (466–502 a.a.) were constructed by PCR amplification of the human liver cDNA libraries. Wild-type pACT2-Daxx was used as a template to construct Daxx deletion mutants, pACT2-D1 (1–282 a.a.), pACT2-D2 (245–508 a.a.), pACT2-D3 (342–625 a.a.), pACT2-D4 (607–740 a.a.), and pGEX-KG-D1-S (46–75 a.a.). Mutations were created by an overlap extention PCR method [Pan and McEver, 1993].

For sumoylation assays in vitro, SUMO and inactive SUMO-Δ were subcloned into NH_2-terminal His-fusion pET-28a expression vector. Daxx and its deletion mutants were subcloned into NH_2-terminal glutathione-S-transferase (GST)-fusion pGEX-KG vector using appropriate oligonucleotides. SUMO and inactive SUMO-Δ deletion mutants were subcloned into NH_2-terminal fusion pRED vector and Daxx and its deletion mutants were subcloned into NH_2-terminal fusion pEGFPC1/2 vectors using appropriate oligonucleotides for in vivo fluorescence microscopy assays. All constructs used in this study were restriction mapped and sequenced.

Yeast Transformation and β-Galactosidase Assay

Yeast strain CG-1945 was co-transformed with a possible pair of pAS2 and pACT2 constructs and analyzed for the interaction. The β-galactosidase assays have been described previously [Ryu et al., 2000].

Sumoylation Assays In Vitro

Sumoylation assays developed by Tatham et al. [2001] have been applied in this study. Plasmids encoding pGST-SAE1/SAE2 and pGST-Ubc9 were kindly provided by Prof. R.T. Hay (University of St. Andrews, UK). The 10 μl reactions were incubated for 3 h at 37°C, then

stopped by adding 1/5 vol 10% SDS, boiled and separated by SDS–PAGE. Sumoylation products were discerned by Coomassie blue staining or immunoblot analysis.

Cell Culture and Transfections

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 3.7 g/L sodium bicarbonate (Amresco), 2 mM L-glutamine (Hyclone), 50 mg/L gentamicin (Invitrogen), and 1% penicillin and streptomycin (Invitrogen), and supplemented with 10% bovine calf serum (Hyclone) in a 5% CO_2 incubator. Transfection was performed using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. Briefly, 10 μl Superfect was mixed with 2 μg of DNA in a serum free Opti-MEM medium (Invitrogen) and added to approximately 1×10^6 cells. Two hours after transfection, the medium was removed and cells were cultured in fresh DMEM with 10% bovine calf serum. Transfected cells were used for observing the protein expressions and cellular localizations by fluorescence/confocal microscopy, Western blotting, and immunoprecipitation assays.

Fluorescent/Confocal Microscopy

pEGFP-Daxx, their mutants, as well as pDsRFP-SUMOs, and C-terminal GG deleted mutants were transfected in HeLa cells and incubated for 12 h. Cells were washed with PBS three times and fixed with 4% paraformaldehyde followed by 0.4% Triton X-100 (ACROS) with 2% FBS (HyClone) in PBS and staining nuclei with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma) for 5 min, washed with PBS three times. Cellular localizations of expressed proteins were examined with the fluorescence/confocal microscope (AXIOskop2$^+$FL, Zeiss/FluoView 500, Olympus), and images were processed with Adobe photoshop software. For each experiment, at least 200 cells were examined.

Immunoblot Analysis

Cells were lysed with ice cold mammalian lysis buffer. The cell lysates containing 1,000 μg proteins were immunoprecipitated with monoclonal mouse anti-GFP (SC-9996) from Santa Cruz, monoclonal mouse living colors-DsRed antibody (8374-2) and polyclonal rabbit living colors-DsRed antibody (632397) from Clontech, and monoclonal mouse anti-actin antibody (MAB1501) from Chemicon, and subjected to electrophoresis. Proteins were immunoblotted with appropriate antibodies and detected with the Western lightning chemiluminescence reagent plus (PerkinElmer life science).

RESULTS

Yeast Two Hybrid Interactions

Using SUMO as bait in yeast two hybrid assays, the C-terminal Daxx fragment was first identified from human liver cDNA libraries. Four Daxx deletion mutants (D1, D2, D3, and D4) (Fig. 1A) were then constructed for further identification of the interaction domains of Daxx with SUMO. Cotransformants were analysized by in vivo filter assay (Table I). The interaction intensities between active SUMO and Daxx fragments were further examined for the β-galactosidase activities using ONPG as a substrate (Fig. 1B). Interaction between pAS2-1-p53 and pACT2-SV40 was used as a positive control and the empty vectors of pAS2-1and pACT2 were used as a negative control.

Table I shows that active SUMO-1 and SUMO-2 have positive filtered assay (indicated as + in Table I) with full length Daxx, N-terminal D1 and C-terminal D4 fragments, but not D2 and D3. The β-galactosidase activities between SUMO-2 and full length of Daxx at 90%, D1 at 98%, and D4 at 121% (shown in Fig. 1B) were found to be similar to that of SUMO-1 and full length Daxx (taken as 100% interaction). However, it was decreased to 25% between SUMO-1 and D1, but increased to about 200% between SUMO-1 and D4. Distinctive β-galactosidase activities suggested that strong interactions between SUMO-2 and D1 as well as SUMO-1 and D4 occurred. Inactive SUMO (SUMO-Δ), a sumoylation-incompetent mutant, was used to suppress the formation of sumoylation linkages in the in-yeast interactions. Positive filter assays (Table I) were obtained from the interactions between SUMO-2-Δ and full length Daxx or D1 deletion mutant. Similarly, SUMO-1-Δ also positively interacted with the full length Daxx or D4 mutant. It further demonstrated that strong non-sumoylation binding interactions exist between SUMOs and Daxx, particularly for SUMO-2 and D1 fragment, and for SUMO-1 and D4 fragment. The fact that D1-K^{60}R mutant, a D1 mutant of which the lysine of amino acid

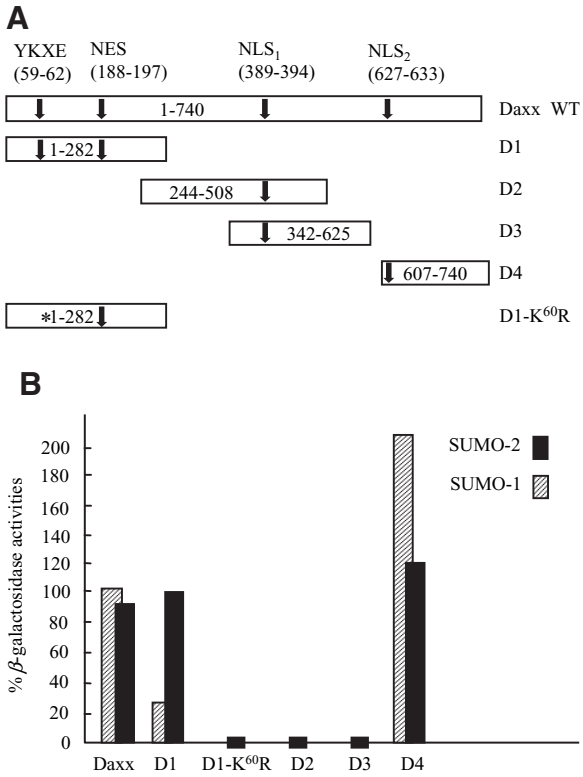

Fig. 1. The interactions between SUMO and Daxx by yeast two hybrid assays. **A**: Schematic representation of wild-type Daxx and its deletion fragments, designated D1, D2, D3, and D4, and a site mutation D1 fragment, D1-K^{60}R, are shown. Amino acid sequences of the deletion fragments are in blocks and those of the related functional motifs in brackets. The N-terminal deletion mutant (D1) contains a sumoylation consensus sequence YKXE and a NES-like motif when both D2 and D3 contains a nuclear localization signal NLS_1 and the C-terminal fragment (D4) contains another nuclear localization signal NLS_2. **B**: β-galactosidase assays of SUMO and Daxx. Plasmids pACT2 encoding Daxx wild-type and deletion mutants, illustrated in Figure 1A, are co-transformed with a possible pair of pAS2-SUMO-2 (or SUMO-1) in yeast strain CG-1945 and analyzed for the interaction. Results of the filter assays are shown on Table I. The β-galactosidase assays were described previously [Ryu et al., 2000]. NES, nuclear export signal; NLS, nuclear localization signal.

residue 60 (K^{60}) of the N-terminal sumoylation consensus sequence (^{57}YKXE^{62}) has changed to arginine, terminating its interactions with SUMO in yeast further suggests that K^{60} is an important interacting residue for positive yeast two hybrid interactions between SUMO and D1 fragment (Table I). Whether K^{60} residue of Daxx is a sumoylation site for SUMO could not be ascertained from the yeast two-hybrid interactions.

In Vitro Sumoylation Assays

The fact that the N-terminal (D1) and the C-terminal (D4) deletion mutants interact positively with SUMO (Table I) indicates that both fragments may contain sumoylation sites. Sumoylation assays in vitro were applied to manifest sumoylation between SUMO and D1 or D4. Active His-SUMO and inactive His-SUMO-Δ reacted with the GST-fused D1 or D4, and then reaction products were analyzed by Western blotting with polyclonal anti-GST or anti-D4 antibodies. The in vitro sumoylation complexes of SUMO and D1 could not be identified by immunoblots with GST-antibodies. This was due to the fact that non-specific proteins migrated at the same position on the gel after electrophoresis and also due to the fact that the D1-specific antibodies were not available. However, the sumoylation complexes of D4 and active SUMO-2 or SUMO-1 (lanes 4 and 6 of Fig. 2, respectively) were obtained, but not using inactive SUMO-2-Δ and SUMO-1-Δ (lanes 5 and 7, Fig. 2, respectively). This indicates that sumoylation sites exist on the D4 fragment. Therefore, three single-site mutants (D4-K^{630}A, D4-K^{631}A, and D4-K^{634}A), a double-site mutant (D4-$K^{630, 631}$A), and a triple-site mutant (D4-$K^{630, 631, 634}$A) of GST-fused Daxx C-terminal mutant proteins (constructs shown in Fig. 3A) were applied to identify the sumoylation sites on the C-terminal Daxx.

TABLE I. Interaction of SUMOs With Daxx Deletion Fragments by Yeast Two Hybrid Filter Assays

	Yeast two hybrid filter assays			
(Amin acids)	SUMO-2	SUMO-2-Δ	SUMO-1	SUMO-1-Δ
Daxx (1–740)	+	+	+	+
D1-K^{60}R (1–282)	–	–	–	–
D1 (1–282)	+	+	+	–
D2 (244–508)	–	–	–	–
D3 (342–625)	–	–	–	–
D4 (607–740)	+	–	+	+

SUMO-2-Δ, inactive SUMO.

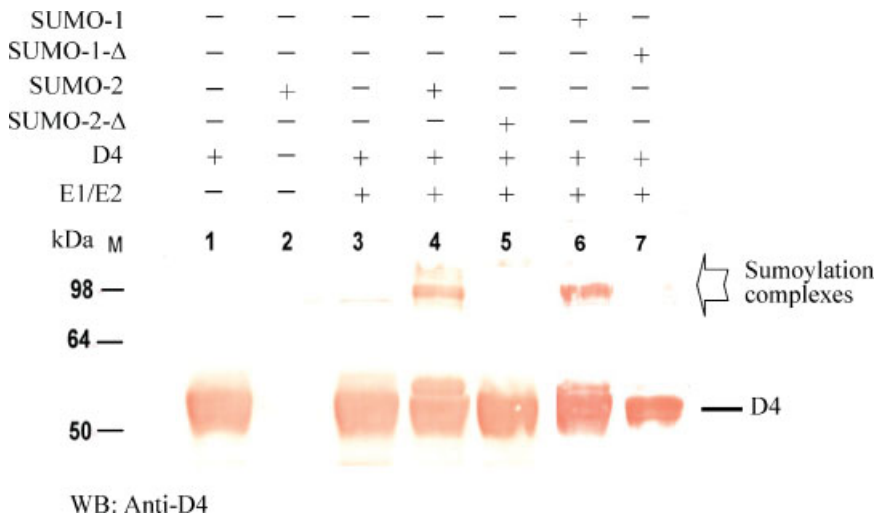

Fig. 2. In vitro SUMO conjugation assays on the C-terminal D4 deletion mutants. In vitro symoylation reactions contained 10 µg GST-Daxx deletion fragments, 10 µl ATP regenerating system (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase and 0.6 U/ml inorganic pyrophosphatase), 5 µg purified His-SUMO and 0.65 µg purified, recombinant GST-Ubc9 (E2) and 0.12 µg purified, recombinant GST-SAE1/2 (E1). The 10 µl reactions are incubated for 3 h at 37°C, then stopped by adding 1/5 vol. 10% SDS, boiled and separated by SDS–PAGE. Sumoylation products are visualized by Western blot with rabbit polyclonal anti-D4 antibody developed by our laboratory.

Sumoylation complexes of His-SUMO-2 or SUMO-1 with GST-D4-K^{630}A, GST-D4-K^{631}A, GST-D4-$K^{630, 631}$A, and GST-D4-$K^{630, 631, 634}$A mutants were shown in Figure 3B. They were analyzed by Western blotting using polyclonal anti-D4 antibodies (panel a and c, Fig. 3B), polyclonal anti-SUMO-2 antibodies (panel b, Fig. 3B) and monoclonal anti-His antibodies (panel d, Fig. 3B). Comparing with that of GST-D4 proteins (lane 3, Fig. 3B), a decreasing trend of sumoylation products (lanes 4, 5, 7, and 8, Fig. 3B) was observed. This indicates K^{630} and

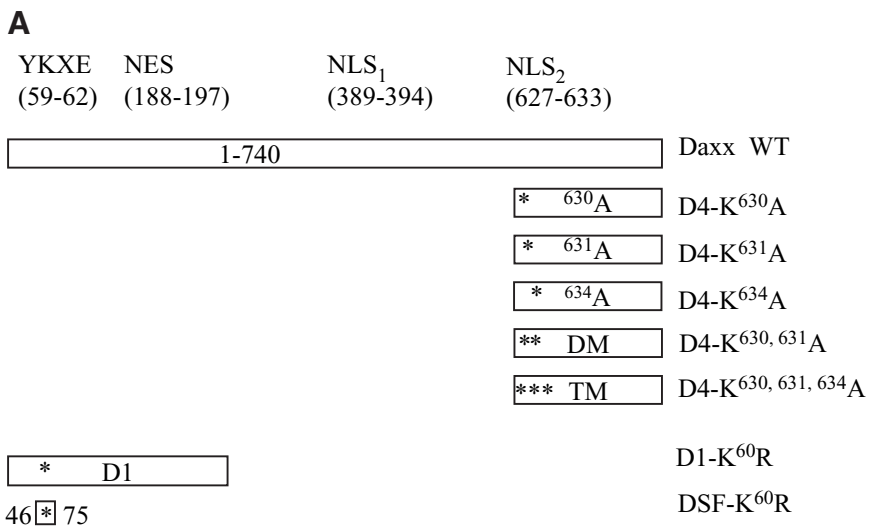

Fig. 3. Lysine 60, Lysine 630, and 631 amino acid residues are the sumoylation sites on Daxx. **A**: Schematic of GST-tagged D4 and GST-tagged D1 mutants are shown with mutated sites indicated by asterisks. YKXE, sumoylation consensus sequence; NES, nuclear export signal like motif; NLS, nuclear localization signal; WT, wild-type; DM, double mutants; TM, triple mutants; DSF, D1 short fragment. **B**: Methods of sumoylation assay in vitro described in Figure 2. Proteins of His-SUMO-2 (and His-SUMO-1) and various GST-tagged D4 mutants (Fig. 3A) were applied on sumoylation assay in vitro. Sumoylation complexes separated by SDS–PAGE, blotted with rabbit polyclonal anti-D4 antibody, anti SUMO-2 or anti-His antibody were detected with the Western lightning chemiluminescence reagent. **C**: His-SUMO-2 (and His-SUMO-1) and GST-tagged DSF and its mutants (Fig. 3A) were applied on sumoylation assay in vitro. Sumoylation complexes were immunobloted with rabbit polyclonal anti-GST antibody when GST-p53F (377-393 a.a.) served as a positive control of sumoylation assay.

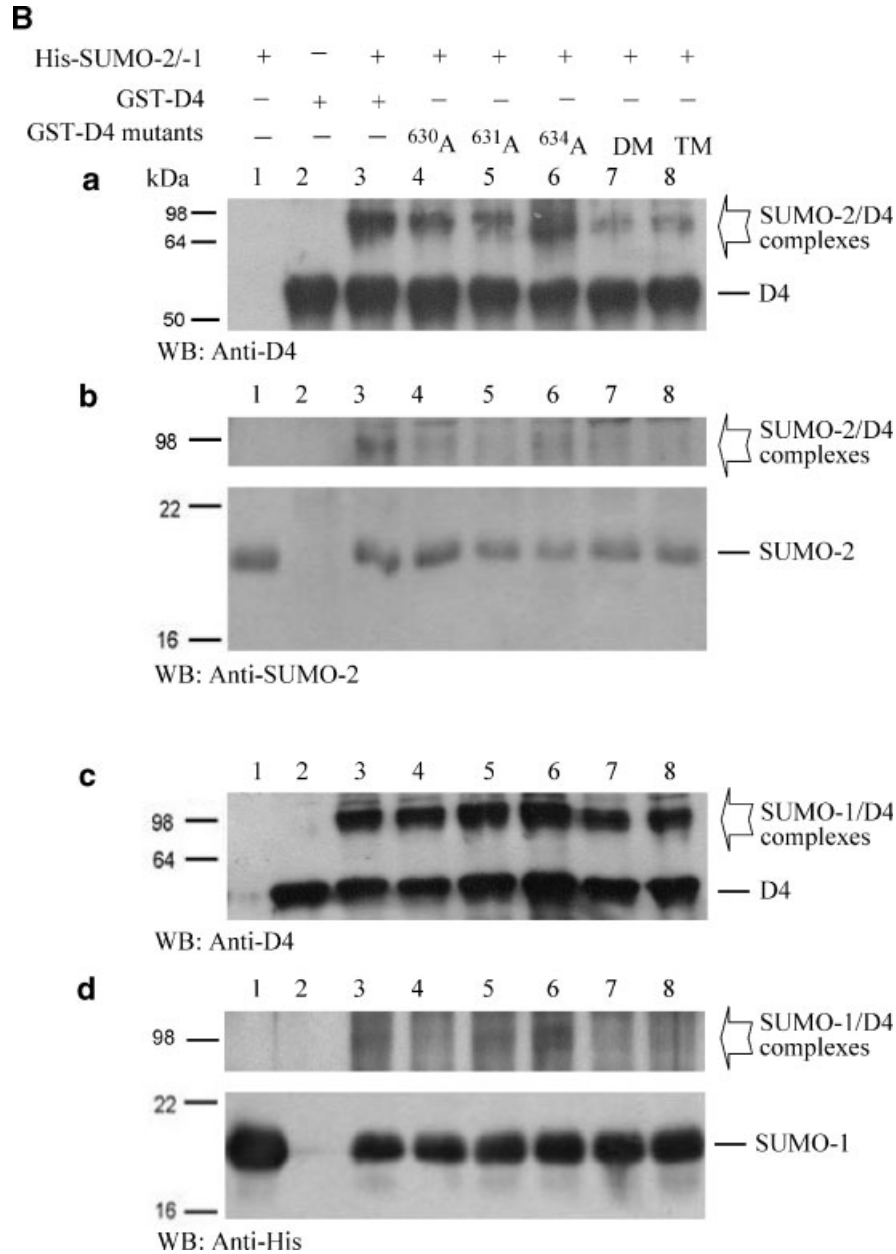

Fig. 3. (*Continued*)

K^{631} are the major SUMO modification sites on the C-terminal Daxx for both SUMO-1 and SUMO-2. Jang et al. [2002] have suggested that K^{630} and K^{631} are the major sumoylation sites of Daxx for SUMO-1 by sumoylation assays in vivo. Current results confirm that the two major sumoylation sites of Daxx are indeed located at the lysine 630 and 631 amino acid residues by in vitro sumoylation assay for not only SUMO-1 but also SUMO-2.

Since D1-K^{60}R mutant has terminated its interactions with SUMO in yeast, indicated as – in Table I, it is likely that sumoylation occurs between SUMO and D1. As previously described, in vitro sumoylation assays could not identify the sumoylation complexes of D1. Therefore, a shorter peptide fragment of Daxx (DSF), amino acid residues 46–75 (constructs shown in Fig. 3A) was used to avoid the interference from non-specific proteins. Small amount of SUMO-1 and SUMO-2 complexes (lanes 1 and 4, Fig. 3C) were observed on DSF, but not on DSF-K^{60}R mutant (lanes 2 and 5, Fig. 3C). The indication is that K^{60} is also a sumoylation site. However, the product yields suggest that K^{60} is a minor sumoylation site,

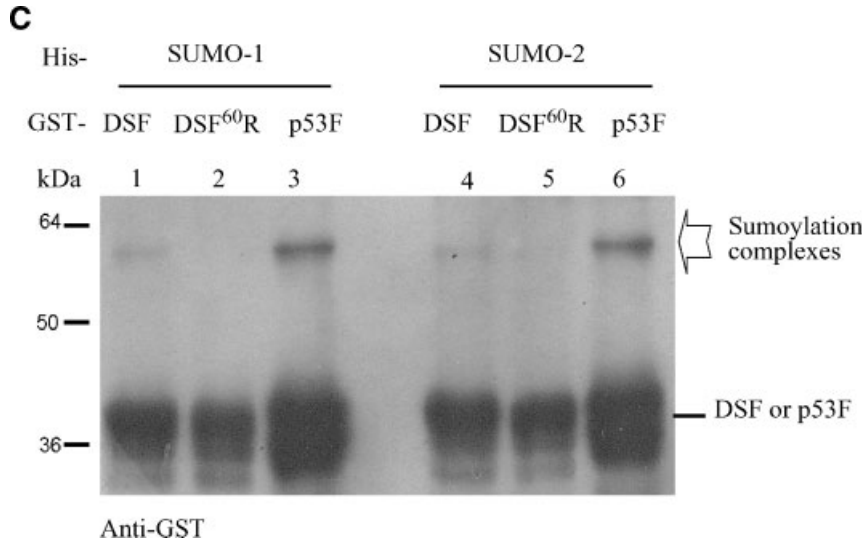

Fig. 3. (*Continued*)

whereas K^{630} and K^{631} are the major sites for SUMO-1 and SUMO-2 modifications.

Cellular Localization of Daxx Deletion Fragments

Four GFP fused Daxx deletion mutants GFP-D1, GFP-D2, GFP-D3, and GFP-D4, as shown in Figure 1A, were transfected into HeLa cells. Results showed that GFP-D2 (Fig. 4Ac) and GFP-D3 (Fig. 4Ad), both fusion proteins contained NLS_1 (amino acids 389–394), were found to diffuse in cytoplasm. However, the fact that N-terminal GFP-D1 (amino acids 1–282) (Fig. 4Ab) and C-terminal Daxx GFP-D4 (amino acids 607–740) (Fig. 4Ae) were localized in nucleus as that of full length Daxx (amino acids 1–740) (Fig. 4Aa) suggests that these two deletion mutants contain the functional NLS motifs.

Cytoplasmic Mislocalization of Daxx Mutants

A consensus sumoylation site K^{60} and a leucine-rich NES-like motif (^{188}IXXLXXLLXL^{197}) found on the N-terminal D1 (Fig. 1A) indicates that they are associated with nuclear translocalization. Single and triple-site mutants were used to examine whether K^{60} or the NES-like motif mediates the nuclear transport of Daxx. Single-site mutants have amino acid altered on both the sumoylation site K^{60} (GFP-D1-K^{60}R) and NES-like motif of D1 (GFP-D1-I^{188}A, GFP-D1-L^{191}A, GFP-D1-L^{194}A, GFP-D1-L^{195}A, and GFP-D1-L^{197}A). Triple-site mutants were prepared by having leucine of 191, 194, and 195 amino acid residues mutated to alanine on both the D1 (GFP-D1-NES^{mut}) fragments and full length Daxx (GFP-Daxx-NES^{mut}). Similarly, triple-site mutants ($K^{630,\ 631,\ 634}$A) on both the C-terminal D4 (GFP-D4-$NLS_2{}^{mut}$) and full length Daxx (GFP-Daxx-$NLS_2{}^{mut}$) were created in order to identify the nuclear translocation function of NLS_2 motif (^{627}PPCKKSRK^{634}). The mutants are illustrated schematically in Figure 5A and the GFP-Daxx fusion proteins of transfected cells analyzed by anti-GFP antibody are shown in Figure 5B.

The nuclear localization of the GFP-D1-K^{60}R (shown in Fig. 5Cb) suggests that the N-terminal consensus sumoylation site K^{60} is not associated with the nuclear transport of D1. Except for some of the GFP-D1-L^{194}A proteins (Fig. 5Ce), all D1 single site mutants (Fig. 5Cb–Ch) were localized in nucleus as that of the wild-type Daxx (Fig. 5Ca). In fact, the GFP-D1-L^{194}A proteins mislocalized in cytoplasm (Fig. 5Ce) were only observed in 50% of the transfected cells. Evidence is compelling that the leucine of amino acids residue 194 is involved in the nuclear transport of D1. Triple-site (191, 194, and 195 a.a.) mutant of D1, GFP-D1-NES^{mut} including leucine 194, mislocalized in cytoplasm on all transfected cells, as shown in Fig. 5Ci, further demonstrates the involvement of NES-like motif on the nuclear transport of D1. Similarly, the triple-site mutant of full length Daxx, GFP-Daxx-NES^{mut}, was mislocalized in cytoplasm on all transfected cells (Fig. 5Cj). This further supports the association of NES-like motif with nuclear translocalization.

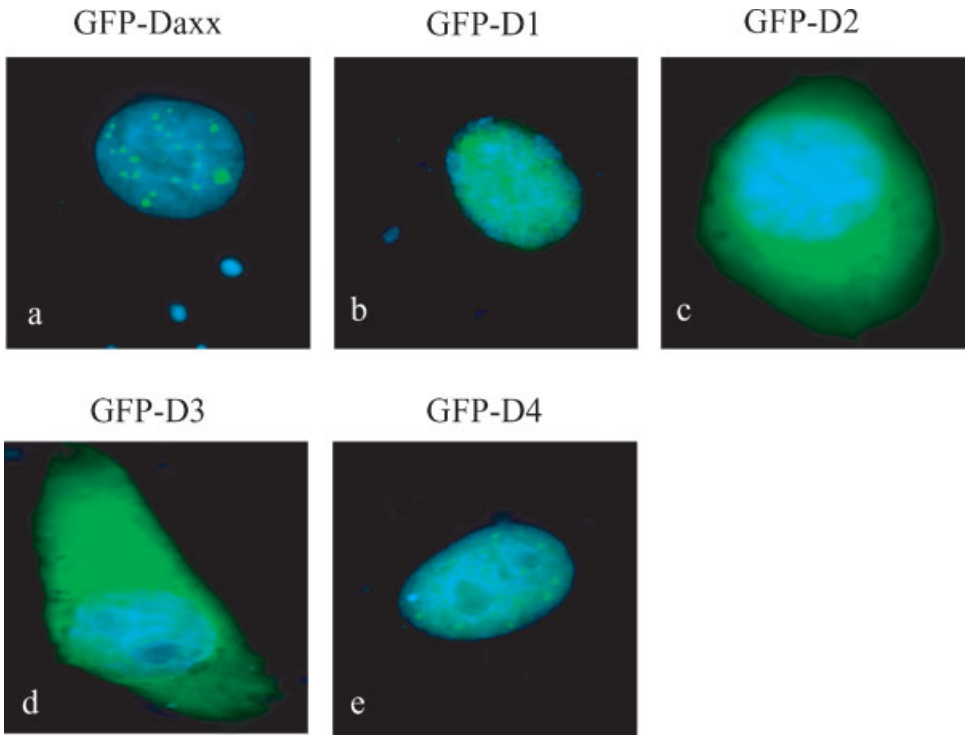

Fig. 4. Fluorescence microscopy reveals D1 and D4 deletion fragments localized in nucleus. The green fluorescent proteins (GFP) fused Daxx and its deletion fragments transiently expressed in HeLa cells after12 h incubation were observed by fluorescence microscopy. The fusion proteins were revealed by the intrinsic green fluorescence of GFP and the nuclei visualized by DAPI staining. The wild-type Daxx (GFP-Daxx) containing YKXE, NES-like motif, NLS_1, and NLS_2 motifs (**a**), the N-terminal deletion mutant D1 (GFP-D1) containing YKXE and NES-like motif (**b**), GFP-D2 deletion mutant containing NLS_1 (**c**), GFP-D3 deletion mutant also containing NLS_1 (**d**), and the C-terminal deletion mutant (GFP-D4) containing NLS_2 (**e**) were merged images.

The N-terminal NES-like motif thus appears to have certain functions in mediating the nuclear transport of D1 as well as of Daxx.

On the other hand, proteins of NLS_2 triple-site (630, 631, and 634 amino acids) mutants GFP-D4-NLS_2^{mut} and GFP- Daxx-NLS_2^{mut}, shown in Figure 5Ck and 5Cl, respectively, have dispersed in cytoplasm on all transfected cells. It is, therefore, likely that the C-terminal NLS_2 motif of Daxx is responsible for the nuclear localization of the C-terminal D4 and for the full length Daxx.

Nuclear Relocalization of Daxx Mutants

It is found that RFP-SUMO-2/-1 fusion proteins localized in nucleus have formed nuclear dots (indicated as N in Table II and Fig. 6Aa,Ac) while the inactive RFP-SUMO-2-Δ proteins were diffused in cytoplasm (indicated as C in Table II and Fig. 6Ab,Ad). The transfected RFP-SUMO fusion proteins analyzed with anti-RFP or anti-SUMO-2 antibodies are shown in Figure 6B with lane 3 showing active SUMO-1 and lane 4 the inactive one; lanes 5 and 9 showing active SUMO-2; and lanes 6 and 10 the inactive SUMO-2.

For further examination of the in vivo interactions between SUMO-2 and Daxx, active SUMO-2 (RFP-SUMO-2) were cotransfected with GFP-Daxx, D1 as well as D4 in HeLa cells. Results (shown in Table II) are consistent with yeast two hybrid interactions in vivo (shown previously in Table II). Active SUMO-2 (RFP-SUMO-2) colocalized with Daxx in nucleus and formed nuclear dots were also observed (indicated as N in Table II and shown in Fig. 7a). Similar images were obtained on the cotransfection of RFP-SUMO-2 and D1 or D4. As cotransfection of RFP-SUMO-2 with Daxx mutants (GFP-Daxx-NES^{mut}, GFP-Daxx-NLS_2^{mutm}, and GFP-DaxxK^{60}R-NLS_2^{mut}), some cytoplasm mislocalized GFP-Daxx mutants were colocalized in nucleus with SUMO-2 as indicated C/N in Table IV and Figure 7b,d, respectively. To investigate if sumoylation was required for the nuclear relocalization of Daxx mutants, inactive

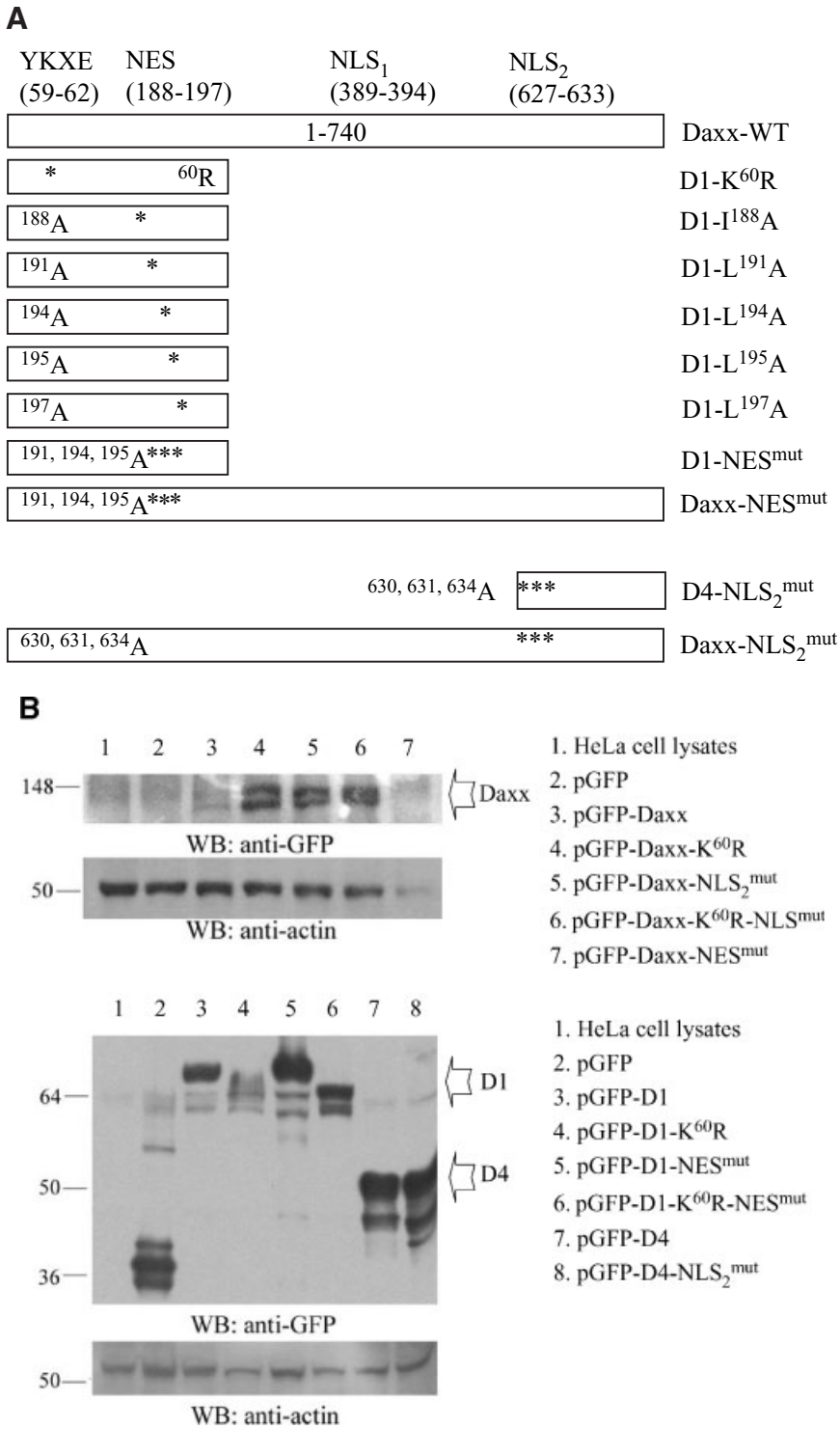

Fig. 5. The NES-like motif and NLS_2 motif are required for the nuclear localization of Daxx. **A**: Schematic representations of Daxx mutants for fluorescent localization assays are shown. The mutated sites are indicated by asterisks. WT, wild-type; YKXE, sumoylation consensus sequence; NES, nuclear export signal-like motif; NLS_2, nuclear localization signal 2. **B**: Western blots of transfected GFP-fusion proteins. Immunoblot of total protein lysates collected from cell populations transfected with plasmids are indicated on the right of Figure 5B. GFP-fused full length Daxx (upper panel) and its deletion mutants (lower panel) were detected with an antibody specific for GFP. **C**: The NES-like motif and the NLS2 motif are required for the nuclear localization of Daxx. Plasmids encoding various GFP-fused Daxx mutants, as shown in Figure 5A, were transfected into HeLa cells and analyzed by fluorescence microscopy after 12 h incubation to identify the NLS motifs. The nuclei were visualized by DAPI staining. The wild-type Daxx (a), the single site mutants of D1 (b–h), the triple-site NES-like motif mutants of D1, and full length Daxx (i and j, respectively) and the triple-site NLS_2 mutants of D4 and Daxx (k and l, respectively) were merged images.

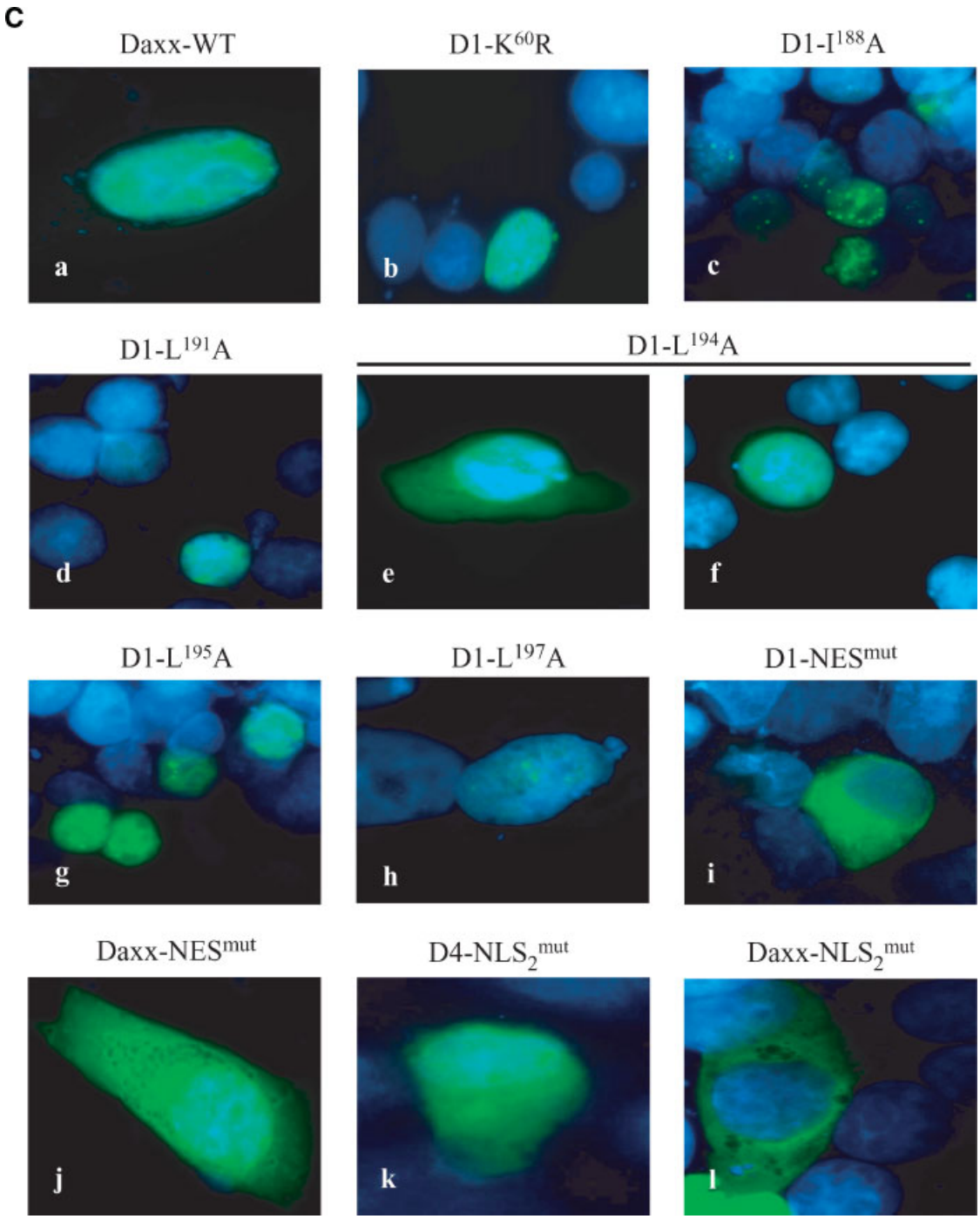

Fig. 5. (*Continued*)

SUMO-2-Δ (or SUMO-1-Δ) was cotransfected with various cytoplasm-mislocalized triple-sites Daxx mutants. RFP-SUMO-Δ and GFP-Daxx mutants were found to disperse in cytoplasm, as indicated C in Table IV and Figure 7e,f. The indication is that sumoylation was needed for the nuclear traslocalization of Daxx.

Inactive SUMO-2-Δ is Associated With Daxx in Nucleus

To examine the in vivo interactions of inactive His-SUMO-2Δ (or His-SUMO-1-Δ) and GST-Daxx, which give positive interactions by yeast two hybrid assays, the inactive RFP-SUMO-2-Δ (or SUMO-1-Δ) and GFP-Daxx were cotrans-

TABLE II. Cellular Localization on Transfection of SUMO and Daxx by Fluorescence Microscopy

		Fluorescent assay in vivo			
		SUMO-2	SUMO-2-Δ	SUMO-1	SUMO-1-Δ
		N	C	N	C
Daxx	N	N	N	N	N
D1	N	N	N	N	C
D4	N	N	C	N	N
Daxx-NES^{mut}	C	C/N	C	C/N	C
Daxx-K^{60}R-NES^{mut}	C	C/N	C	C/N	C
Daxx-NLS$_2$^{mut}	C	C/N	C	C/N	C
Daxx-K^{60}R-NLS$_2$^{mut}	C	C/N	C	C/N	C

SUMO-2-Δ, inactive SUMO; N, nucleus; C, cytoplasm; Daxx-NES^{mut}, Daxx-K$^{191, 194, 195}$A; Daxx-NLS$_2$^{mut}, Daxx-K$^{630, 631, 634}$A.

fected in HeLa cells. Figure 8Aa,Ab shows the nuclear colocalization and formation of nuclear dots of these two proteins by fluorescence microscopy assays. They are in good accord with those of yeast two hybrid assays. Moreover, the immunoprecipitation of the cotransfected cell lysates with anti-RFP antibody, then Western blotting with anti-GFP antibody shows that GFP-Daxx is associated with inactive RFP-SUMO-2-Δ (or RFP-SUMO-1-Δ) (upper panel, lane 6 and 3, respectively), as that of active RFP-SUMO-2 (or RFP-SUMO-1) (upper panel, lane 5 and 2, respectively). Similarly, when the cotransfected cell lysates were immunoprecipitated with anti-GFP antibody and Western blotted with anti-RFP antibody, the inactive RFP-SUMO-2-Δ (or RFP-SUMO-1-Δ) was observed on the lower image of Figure 8B lane 4 and 2, respectively. The positive controls of the associations between active RFP-SUMO-2 (or RFP-SUMO-1) and GFP-Daxx are shown on the lower images of Figure 8B, lane 3 and 1.

DISCUSSION

Yeast Two Hybrid Interactions

Sumoylation assay is a covalent bonding reaction between glycine of the C-terminal SUMO and the lysine of its target protein, while yeast two hybrid assay is the binding interactions between SUMO and Daxx molecules, which may involve a wider range of binding interactions including possibly non-covalent interactions and covalent linkages. Sumoylation between SUMO and Daxx may have occurred during yeast two hybrid interactions; however, the positive filter assays or β-galacsidase assays between SUMO and Daxx could also be achieved through the physical interaction between these two molecules without sumoylation linkages, which is referred to as "non-sumoylation interactions" in this study. There are strong interactions between SUMO-2 and D1 as well as SUMO-1 and D4, as shown on Figure 1B. The interactions were mainly non-covalent illustrated by the positive filter assay when sumoylation-incompetent inactive SUMO-Δ was used (Table I). However, the reason SUMO-1 and SUMO-2 behave differently in yeast two hybrid interactions with D1 or D4 might due to the effects of their variable N-terminus. It has been suggested in the previous studies by Su and Li that although the function of SUMO's N-terminal extension is presently unknown, its characteristics, that is being rich in charged amino acids, glycines and prolines make it an excellent candidate for specific protein–protein interactions. [Su and Li, 2002]

Two Functional Motifs are Required for the Nuclear Transport of Daxx

The fact that the NLS$_1$ on D2 and D3 of Daxx deletion fragments is not a functional nuclear localization signal (Fig. 3c,d) confirms that NLS$_1$ is not a functional motif as proposed by Torii et al. [1999].

A leucine-rich NES-like motif (^{188}IXXLXXLLXL^{197}), termed conveniently as NES, was found on the N-terminal Daxx. Its conserved sequence of leucine residues was similar to that of p53 (^{341}FXXLXXXLXL^{350}) [Stommel et al., 1999]. This motif was not a conventional lysine-rich NLS, but it has become an NLS candidate due to possible interactions with the nuclear pore complexes (NPCs). That the triple-site mutants of D1 (GFP-D1-NES^{mut}) terminate nuclear

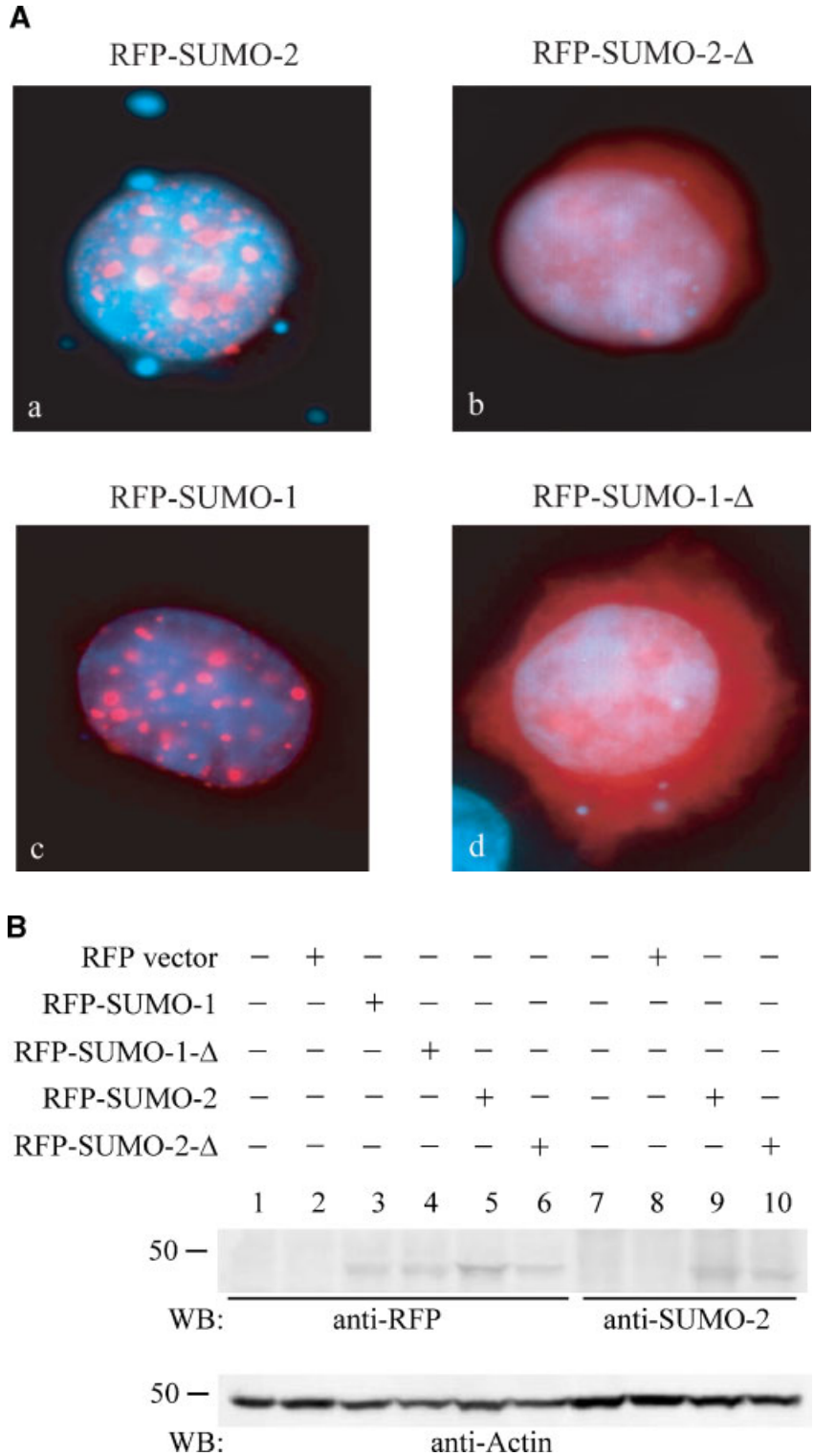

Fig. 6. Inactive SUMO is mislocalized in cytoplasm. **A**: Plasmids encoding red fluorescent proteins fused SUMO (RFP-SUMO-1/-2) or inactive SUMO (RFP-SUMO-1-Δ/-2-Δ) were transfected into HeLa cells. The slide was stained with DAPI and analyzed by fluorescence microscopy. These figures are merged. **B**: RFP-SUMO fusion proteins were detected with anti-RFP antibody (lanes 3–6) and RFP-SUMO-2/-2-Δ were detected with anti-SUMO-2 antibody (lanes 9–10).

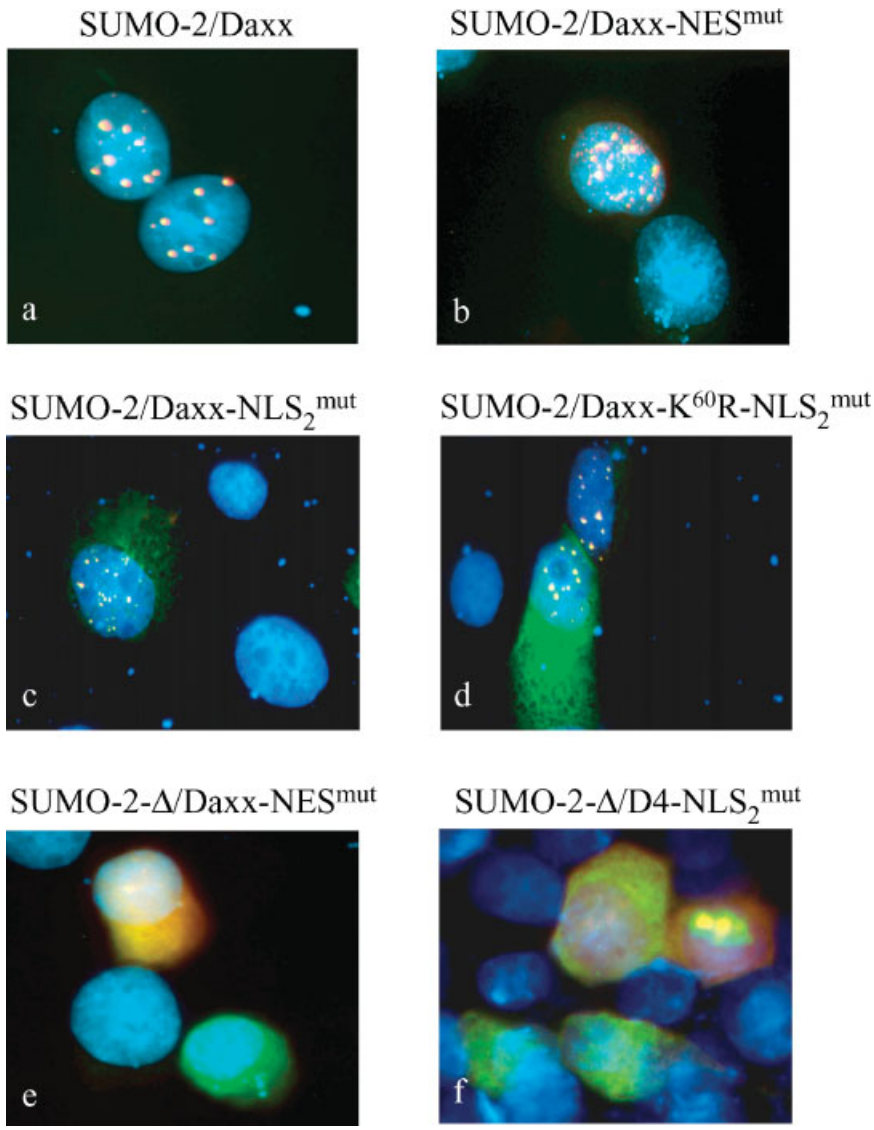

Fig. 7. Sumoylation enhances the nuclear localization of Daxx mutants. HeLa cells were cotransfected with GFP-Daxx (**a**) or various Daxx mutants (**b**–**d**) and SUMO-2 (a–d) or inactive SUMO-2-Δ (**e**, **f**), as indicated above the images. The transfected cells were incubated for 12 h before analyzed with fluorescence microscopy. The yellow color in the merged image is due to colocalization of green fluorescent protein fused Daxx and red fluorescent protein fused SUMO.

transport (Fig. 5Bi) confirm that the NES motif is required in mediating the nuclear transport of D1.

C-terminal Daxx has indeed been suggested to be a nuclear localized protein before [Pluta et al., 1998; Hollenbach et al., 1999; Jang et al., 2002]. The nuclear translocalizations of the C-terminal Daxx could have been due to the function of proposed C-terminal NLS_2 (amino acids 627–633). However, it was regarded by Jang et al. [2002] as non-functional (amino acids 627–633) by cotransfection of double-site mutant Daxx-$K^{630,\ 631}$A and PML colocalized in nucleus. The termination of nuclear transport of the C-terminal fragment D4 (shown in Fig. 5Bk) by triple-site mutants $K^{630,\ 631,\ 634}$A of D4 (GFP-D4-NLS_2^{mut}) has established the nuclear transport function of NLS_2 on the C-terminal Daxx.

Dispersion of mutants of full length Daxx (GFP-Daxx-NES^{mut} and GFP-Daxx-NLS_2^{mut}) on either N-terminal NES-like motif or C-terminal NLS_2 motif in cytoplasm (shown in Fig. 5Bj,Bl) leads to the conclusion that both

NES-like motif and NLS_2 motif are required for the nuclear transport of Daxx.

SUMOs Enhance the Nuclear Transport of its Target Protein

Investigating the role of SUMO-2 on the nuclear transport of Daxx has demonstrated that relocalization of cytoplasm mislocalized Daxx mutants occurs during cotransfection with SUMO-2. It can be seen that almost all GFP-Daxx-NES^{mut} proteins were colocalized in nucleus with SUMO-2 (Fig. 7b), whereas GFP-Daxx-$NLS_2{}^{mut}$ and GFP-DaxxK^{60}R-$NLS_2{}^{mut}$ proteins were partially colocalized in nucleus with SUMO-2 (Fig. 7c,d, respectively). This could be explained by intact major sumoylation sites K^{630} and K^{631} that existed in GFP-Daxx-NES^{mut} proteins but that were lacking in GFP-Daxx-$NLS_2{}^{mut}$ and GFP-DaxxK^{60}R-$NLS_2{}^{mut}$ proteins. Relocalization of GFP-Daxx-$NLS_2{}^{mut}$ proteins has probably occurred through interacting with SUMO-2 on minor sumoylation sites K^{60}. A mutated K^{60} should not have relocalized GFP-Daxx-K^{60}R-$NLS_2{}^{mut}$ proteins; relocalization could then have taken place through non-sumoylation interactions or unidentified minor sumoylation sites on the C-terminal of Daxx. Unidentified minor sumoylation sites were indicated by the observation of sumoylation complexes from in vitro sumoylation of TM ($K^{630,\ 631,\ 634}$A) on D4 (Fig. 3B). Relocalization of Daxx mutants (Figs. 7e,f) blocked in inactive SUMO-2-Δ, however, suggests sumoylation enhances cytoplasmonuclear translocalization of Daxx.

Interactions between RanGAP1 and NPC enhanced by sumoylation RanGAP1 [Matunis et al., 1996; Tatham et al., 2005; Reverter and Lima, 2005] suggest the role of sumoylation in regulating protein–protein interactions in cytoplasm. Similar to RanGAP1, Daxx may also have sumoylated in cytoplasm before interacting with NPC. That is why GFP-Daxx-NES^{mut} and GFP-Daxx-$NLS_2{}^{mut}$ were localized in cytoplasm with sumoylation defective SUMO-2-Δ (Figs. 7e,f). Nuclear localization of sumoylated Caspase 8 was studied by Besnault-Mascard et al. [2005]. They reported that Caspase 8 was cytoplasm localized while the sumoylated Caspase 8 was found only in nucleus. This is similar to Daxx mutants; the nuclear transport of Caspase 8 could have been mediated by sumoylation. Proteins entering nucleus facilitated by sumoylation has been demonstrated by SUMO-1 fused NF-κB essential modulator (NEMO) [Huang et al., 2003] that functions similarly to Daxx. They reported that the wild-type NEMO proteins were localized in cytoplasm whereas the SUMO-1 fused NEMO were found in both cytoplasm and nucleus. It is likely that a new biological role of sumoylation is to regulate the cytoplasmonuclear transport of its target proteins.

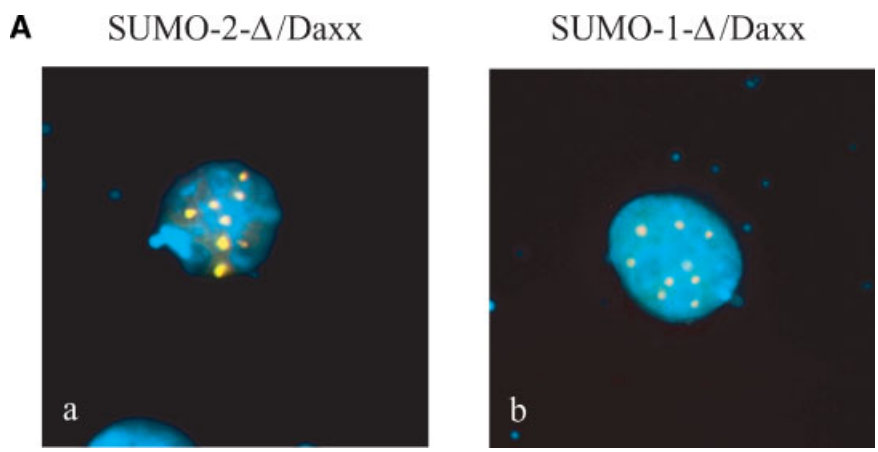

Fig. 8. Inactive SUMO-Δ can be relocalized in nucleus by non-sumoylation interactions with Daxx. **A**: Inactive RFP-SUMO-2-Δ and GFP-Daxx are colocolized in nucleus. The inactive RFP-SUMO-2-Δ (or RFP-SUMO-1-Δ) were cotransfected with GFP-Daxx in HeLa cells. The transfected cells were incubated for 12 h before analysis by fluorescence microscopy. The yellow color in the image is due to colocalization of GFP-fused Daxx and rRFP-SUMO (Fig. 8Aa,Ab). **B**: Non-sumoylation interactions occur between inactive SUMO-Δ (or active SUMO) and Daxx. HeLa cells were cotransfected with RFP-SUMO (or inactive SUMO-Δ) and GFP-Daxx as indicated. Approximately 1,000 μg of total cell extracts were subjected to IP with anti-RFP antibody followed by WB with anti GFP antibody (upper panel) and vise versa (lower panel).

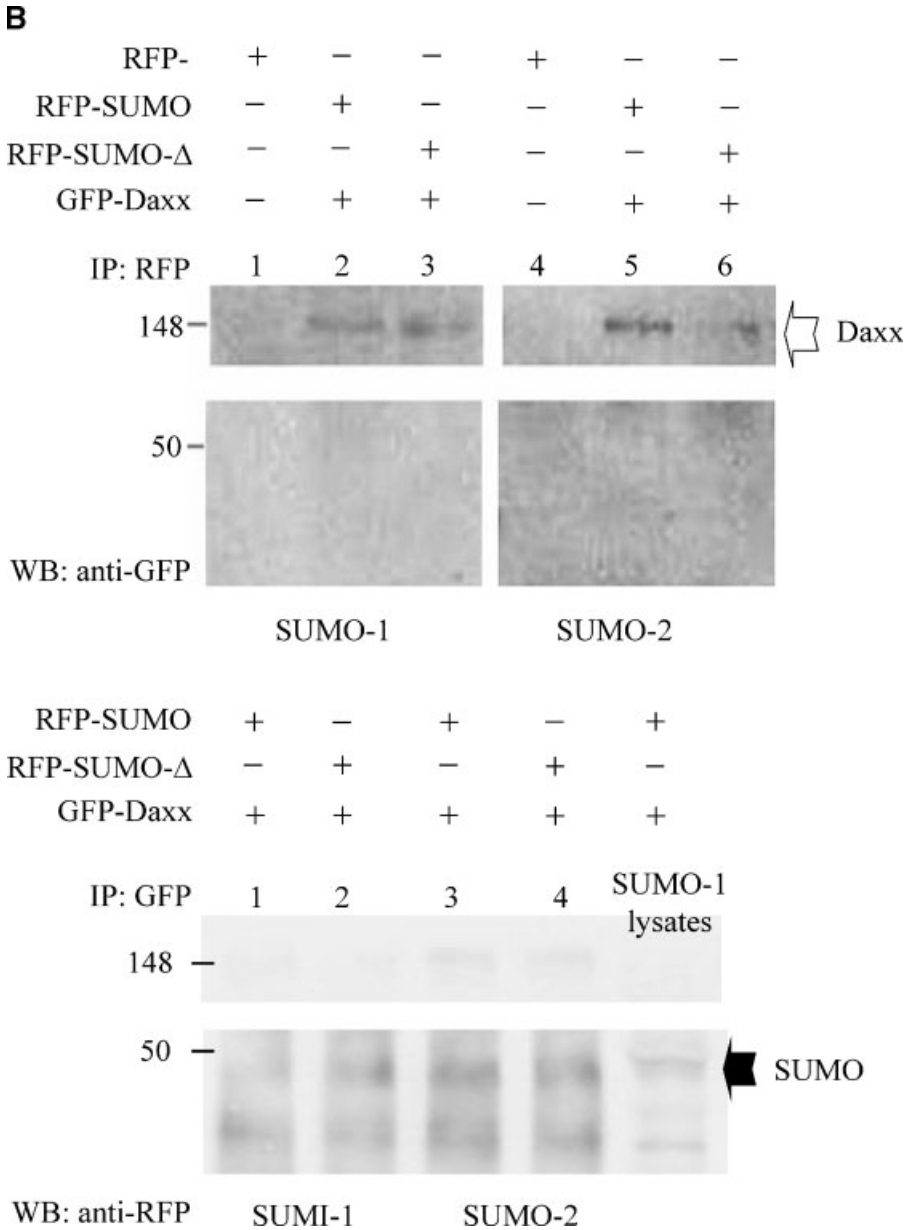

Fig. 8. (*Continued*)

Non-Sumoylation Interactions are Found Between Inactive SUMO-Δ and Daxx

Forming SUMO–SUMO complexes or SUMO-target protein complexes appears necessary for nuclear translocation since inactive SUMO-Δ, being unable to sumolyate (lane 5 in Fig. 2), was localized in cytoplasm (Fig. 6Ab). Nevertheless, inactive SUMO-Δ relocalizing to nucleus by its target protein Daxx in fluorescence microscopy assays (Fig. 8Aa,Ab) and giving positive yeast two hybrid interactions (Table I) with Daxx were found consistently in this study. The suggestion is that a significant non-sumoylation interaction has occurred between inactive SUMO-Δ and Daxx. This could have provided a new mechanism, besides sumoylation, that acts on improving the nuclear transport of target proteins by SUMO.

From yeast two hybrid studies and in vivo fluorescent assays, it can be concluded that double glycines on the C-terminus of SUMO and the K^{60} amino acid residue, the NES-like motif as well as the NLS_2 motif of Daxx are critical

areas for non-sumoylation interactions between SUMO and Daxx. Considerable non-sumoylation interactions between SUMO and its target proteins (Fig. 8) may explain why mutating sumoylation sites in Sp100, PML, or p53 did not prevent intranuclear accumulation in transfection experiments [Sternsdorf et al., 1999; Zhong et al., 2000; Lallemand-Breitenbach et al., 2001; Kwek et al., 2001]. Therefore, nuclear localization of Daxx may have been regulated by SUMO through sumoylation as well as non-sumoylation interactions in cytoplasm. Several conclusions can be drawn from this study: (a) two functional motifs, leucine-rich NES-like motif (^{188}IXXLXXLLXL^{197}) and C-terminal NLS_2 (amino acids 627–633), are required for the nuclear transport of Daxx; (b) SUMO enhance the nuclear transport of its target proteins Daxx; and (c) it may be done through the non-sumoylation interaction.

The cytoplasm mislocalized Daxx mutants (Daxx-NES^{mut}, Daxx-$NLS_2{}^{mut}$, and Daxx-K^{60}R-$NLS_2{}^{mut}$) with different sumoylation conditions available may provide us an opportunity of exploring the necessities of sumoylation on Daxx-involved interactions on the Fas induced apoptotic signals or ASK1 apoptotic signals. Therefore, identifying SUMO's involvement on Daxx-related apoptosis and its connection to the degradation of Daxx after transferring apoptotic signals becomes an important task in the future.

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