Journal of Cellular Biochemistry 103:456–470 (2008)

Daxx Contains Two Nuclear Localization Signals and Interacts With Importin α3

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Abstract Daxx plays a major role in several important signaling pathways including transcription and cell death. It has been postulated that Daxx regulates both events from the nucleus; however, the mechanism by which Daxx is localized in the nucleus remains obscure. Here we show that nuclear localization of Daxx is controlled by two independent signals and importin 3. Domain analysis reveals that Daxx contains two separate nuclear localizing domains. Site-directed mutagenesis reveals that the basic aa sequence RLKRK at residues 227–231 (NLS1) is responsible for nuclear localization of N-terminal domain, while aa sequence KKSRKEKK at residues 630–637 (NLS2) is responsible for nuclear localization of the C-terminal domain. Mutations of a NLS consensus sequence RKKRR at residues 391–395 and several other basic aa clusters have no effect on Daxx nuclear localization. In full-length Daxx, NLS1 contributes partially to nuclear localization, while NLS2 plays a major role. Markedly, it is essential to disrupt both NLS1 and NLS2 in order to completely block nuclear localization of the full-length protein and to prevent its association with PML nuclear bodies. Furthermore, Daxx interacts selectively with importin α3 through its NLS1 and NLS2 sequences. Conversely, importin α3 utilizes two NLS-binding sites for Daxx interaction, suggesting that the importin/mediates nuclear import of Daxx. Finally, we show that nuclear localization of Daxx is essential for its transcriptional effects on GR and p53. Together, these data unveil a molecular mechanism that controls nuclear localization of Daxx and support a nuclear role of Daxx in transcriptional regulation. J. Cell. Biochem. 103: 456–470, 2008.

Key words: Daxx; nuclear localization signals; importin α3; GR; p53; PML nuclear bodies

Daxx is an essential protein implicated in regulating transcription and cell death (apoptosis) (see review [Salomoni and Khelifi, 2006]). It was first described as a pro-apoptotic protein associated with the death receptor Fas and Ask1 [Yang et al., 1997; Chang et al., 1998], and a transcriptional regulator interacting with the promyelocytic leukemia protein (PML) [Hollenbach et al., 1999; Ishov et al., 1999; Torii et al., 1999; Li et al., 2000]. Daxx interacts with many other proteins, including the heat-shock protein HSP27 and the Parkinson's disease protein DJ-1, which block the pro-apoptotic function of Daxx [Charette et al., 2000; Junn et al., 2005]. Daxx also interacts with the type II TGF-β receptor, mediating JNK activation and apoptosis [Perlman et al., 2001]. Other evidence also suggests that Daxx may have an anti-apoptotic role, as Daxx knockout or knock-down cells display enhanced apoptosis [Michaelson et al., 1999; Chen and Chen, 2003; Michaelson and Leder, 2003].

The role of Daxx in transcription is well documented as it interacts directly with several transcription factors, including Pax3/5 [Hollenbach et al., 1999; Emelyanov et al., 2002], ETS1 [Li et al., 2000], p53 and family proteins [Kim et al., 2003; Zhao et al., 2003, 2004; Gostissa et al., 2004], Smad4 [Chang et al., 2005], STAT3 [Muromoto et al., 2005], and nuclear receptors for androgen and glucocorticoid (GR) [Lin et al., 2003, 2004; Mizuta and Kuroda, 2004; Obradovic et al., 2004]. In these cases, Daxx acts

Abbreviations used: NLS, nuclear localization signal; PML, promyelocytic leukemia protein; POD, PML oncogenic domains; GR, glucocorticoid receptor; PAH, paired amphipathic helix.

Grant sponsor: National Cancer Institute and National Institutes of Health; Grant number: CA87074.

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Received 27 March 2007; Accepted 18 April 2007
DOI 10.1002/jcb.21408

as a corepressor, inhibiting transcriptional activation [Emelyanov et al., 2002]. Daxx also interacts with transcriptional coactivators like CBP and Skip/NCoA62 [Kuo et al., 2005; Tang et al., 2005], and corepressors like histone deacetylases [Li et al., 2000; Hollenbach et al., 2002], DNA methyltransferase 1-associated protein DMAP1, and TSG101 [Muromoto et al., 2004a,b]. These interactions suggest that Daxx may influence transcription through association with transcriptional coregulators. Furthermore, Daxx also interacts with the interphase centromeric protein CENP-C [Pluta et al., 1998]. Biochemical purification of Daxx reveals the presence of core histones, chromatin-associated protein Dek, and X-linked mental retardation and alpha-thalassaemia syndrome protein ATRX [Hollenbach et al., 2002; Xue et al., 2003; Tang et al., 2004]. These Daxx-containing complexes suggest that Daxx may influence transcription through modulating chromatin structure.

Daxx contains a C-terminal Ser/Pro/Thr (S/P/T)-rich domain, which mediates interactions with most of its partners. This C-terminal domain is frequently used as a dominant negative mutant for studying the function of wild type Daxx [Yang et al., 1997; Chang et al., 1998; Perlman et al., 2001; Wu et al., 2002; Hofmann et al., 2003; Raoul et al., 2005]. Near the center of the protein is a stretch of acidic aas (D/E), mediating interactions with the cytomegalovirus protein pp71 and p53 [Hofmann et al., 2002; Ishov et al., 2002; Zhao et al., 2004; Cantrell and Bresnahan, 2005]. Daxx also contains a central coiled-coil like domain [Pluta et al., 1998], and two N-terminal paired amphipathic helices PAH1 and PAH2 [Hollenbach et al., 1999]. These domains have been shown to mediate protein–protein interactions [Lin and Shih, 2002; Muromoto et al., 2004a,b], but their exact role in Daxx function remains unclear.

Daxx is localized mainly in the nucleus, where it is seen prominently at PML-oncogenic domains (PODs, or PML nuclear bodies) [Ishov et al., 1999; Li et al., 2000]. The association of Daxx with PODs is of clinical relevance, as acute promyelocytic leukemia with t(15:17) translocation causes POD dissociation and release of Daxx into nucleoplasm, while retinoic acid restores PODs and induces cell differentiation [Li et al., 2000]. Furthermore, SUMO-1-modified PML could recruit Daxx to PODs [Ishov et al., 1999; Li et al., 2000], while interaction with CENP-C might cause association of Daxx with interphase centromeres [Pluta et al., 1998; Everett et al., 1999]. Interferon induces expression and translocation of Daxx to PODs [Gongora et al., 2001], and Daxx could also be translocated to the nucleolus by MSP58 [Lin and Shih, 2002]. Moreover, Daxx could shuttle between the nucleus and cytoplasmic compartments. The protein responsible for nuclear export of Daxx was recently identified as CRM1 (chromosomal region maintenance 1), which binds to a Daxx nuclear export signal at residues 566–576 [Song and Lee, 2004]. However, the exact nuclear targeting sequences and importing pathway are currently unknown.

The transportation of large proteins into cell nuclei requires an energy-dependent mechanism (for reviews, see References Goldfarb et al. [2004] and Weis [2003]). One classic pathway of nuclear import is mediated by the importin α/β system. Six human α importins are currently known, including importin α1/Rch1/hSRP1α, α3/Qip1, α4/KPNA3/hSRP1γ, α5/hSRP1, α6 and α7 [Cuomo et al., 1994; Weis et al., 1995; Kohler et al., 1997, 1999; Seki et al., 1997; Takeda et al., 1997; Nachury et al., 1998]. Importin α binds directly to cargo proteins in the cytoplasm, which then interacts with importin β1 at the nuclear pore complex, followed by translocation of the cargo into nucleoplasm [Chi et al., 1995; Gorlich et al., 1995]. Several importin βs can also recognize specific nuclear localization signals (NLSs) directly without importin α [Mosammaparast and Pemberton, 2004; Lee et al., 2006]. Once inside the nucleus, the cargo protein is released from importin in a Ran-GTP-dependent manner, and importin is then exported back to the cytoplasm.

Several prior studies have shown conflicting results regarding the signals that regulate Daxx nuclear localization [Pluta et al., 1998; Torii et al., 1999; Jang et al., 2002]. To clarify the signals responsible for Daxx nuclear localization, we conducted domain localization analysis and mutated potential NLS sequences of Daxx within separated nuclear domains and the full-length protein. We also investigated the role of various importins in mediating nuclear import of Daxx. Our results clearly defined two separate signals as responsible for Daxx nuclear localization, and uncovered a specific

importin as the molecular mediator for Daxx nuclear import.

MATERIALS AND METHODS

Cell Culture and Reagents

HeLa, COS-7, and HEK293 cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 5 μg/ml of gentamycin (Life Technologies, Grand Island, NY). Cell transfection was conducted by the standard calcium phosphate precipitate method. Appropriate numbers of cells (about 75% confluent) were plated in multi-well plates for 12 h, and cells were then incubated with DNA-$CaPO_4$ precipitates for 12 h. Transfected cells were washed three times with PBS, replaced with fresh media, and harvested 36 h afterward. All chemical reagents were of the highest quality available from various commercial sources.

Plasmids

The human Daxx cDNA used in this study was isolated from the HeLa cDNA library [Li et al., 2000]. HA-DaxxF, its site-directed mutants, and other HA-Daxx mutants contain an optimized N-terminal translation start site with an HA tag (MDYPYDVPDY) at the N-terminus. Site-directed mutagenesis was conducted using the QuickChange kit (Stratagene, Inc.). Deletion mutants were generated by PCR using Pfu with primer sets containing restriction sites for subcloning. All mammalian expression vectors were cloned in the pCMX vector, except the HA-Daxx 555-740 deletion mutant and its point mutants are in the pCDG-1 vector (a derivative of pcDNA1). EGFP fusion constructs were cloned in the pEGFP-C1 vector (BD Bioscience, Inc.). GST fusion constructs were cloned in the pGEX-2T vector. GST-importins were kindly provided by Dr. Riku Fagerlund (National Public Health Institute, Helsinki, Finland) [Fagerlund et al., 2005], except for importin α4 (KPNA3), which was isolated in our laboratory from a human placenta cDNA library.

GST Pull-Down Assay

GST pull-down assay was conducted according to a protocol described previously [Harlow and Lane, 1988]. Briefly, 5 μg of GST fusion proteins coupled with glutathione agarose beads were incubated with 5 μl of in vitro translated ^{35}S-labeled protein with moderate shaking at 4°C overnight in binding buffer (20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40, 1 mM DTT, 1 mg/ml BSA). The bound protein was washed three times with the binding buffer and beads were collected by low speed centrifugation. The bound protein was eluted in SDS sample buffer and analyzed by SDS–PAGE and autoradiography.

Immunofluorescence Microscopy

Cells were fixed in 4% paraformaldehyde for 10 min or in cold methanol/acetone (1:1) mixture on dry ice for 1 min. The fixed cells were processed for immunofluorescence staining as previously described [Dyck et al., 1994]. After washing, cells were stained with rhodamine- or fluorescein-conjugated goat anti-rabbit or anti-mouse secondary antibodies. Primary antibodies used in this study include the affinity purified Daxx polyclonal antibody [Li et al., 2000], the PML monoclonal antibody 5E10 [Stuurman et al., 1992], the anti-HA mouse monoclonal (F-7, Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antibody (Medical Biological Lab, Inc.). Cell nuclei were stained with DAPI. The processed cover glasses were mounted on slides with Pro-Long anti-fade reagent (Molecular Probe), and visualized with a Zeiss Axiovert 200 inverted epi-fluorescence microscope. Images were captured with Axiocam and analyzed by Axiovision software. Color images were exported from Axiovision and assembled using Canvas.

Luciferase Reporter Assay

Twelve hours prior to transfection, 2×10^4 cells were plated in each well of 12-well plates. Transfected cells were referred with fresh media and harvested 36–48 h after. For luciferase and β-galactosidase assays, transfected cells in each well were lysed and processed for luciferase and β-gal assay as described [Li et al., 1997]. The luciferase activity was determined with a MLX plate luminometer (Dynex) and normalized with the cotransfected β-galactosidase activity.

RESULTS

Identification of Daxx Nuclear Localizing Domains

To identify domains that are responsible for nuclear localization of Daxx, subcellular

distribution of several Daxx fragments were determined in COS-7 cells (Fig. 1A,B). As expected, full-length Daxx localizes predominantly in the nucleus and forms POD structures at low expression levels. Amino acids (aa) 1–125 fragment localizes mainly in the cytoplasm, indicating that it is not involved nuclear localization. Interestingly, aa 1–267 fragment localizes in the nucleus, even though this region does not contain any predictable NLS. Similarly, aa 216–502 fragment also localizes in the nucleus. In contrast, aa 298–622 fragment distributes evenly throughout the cell, despite the presence of a predicted NLS consensus sequence at residues 391–395. These results suggest that the aa 391–395 motif is not

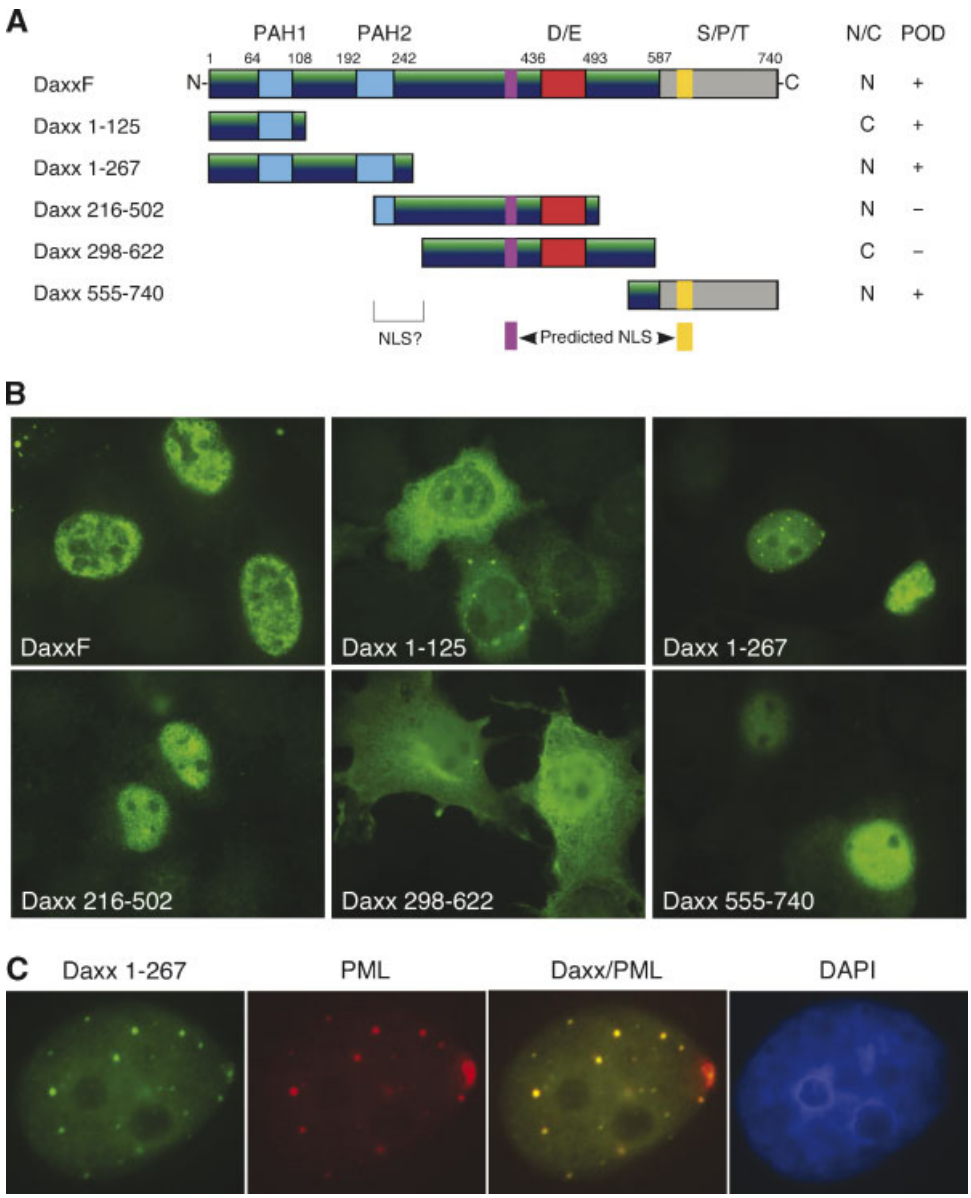

Fig. 1. Identification of Daxx nuclear localizing domains. **A**: Schematic representation of Daxx deletion mutants. Daxx domains and boundary residues are marked on top. Two predicted NLSs at residues 391–395 and 627–634 are indicated at bottom. Nucleocytoplasmic (N/C) distributions of these Daxx domains and their associations with PODs are summarized at the right. PAH, paired amphipathic helix; D/E, Asp/Glu-rich domain; S/P/T, Ser/Pro/Thr-rich domain. **B**,**C**: Immunofluorescence analysis of Daxx fragments. COS-7 cells were transfected with indicated Daxx domains and analyzed by immunofluorescence staining with anti-HA polyclonal antibody (green) and anti-PML monoclonal antibody 5E10, followed by fluorescein (FITC) or rhodamine-conjugated secondary antibody. Colocalization between Daxx 1–267 domain and endogenous PODs is shown in (C). DAPI staining shows the nucleus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

a functional NLS, while there may be a functional NLS between aa 125 and 298. As expected, the C-terminal aa 555–740 fragment containing the second predicted NLS localizes in the nucleus. Together, these results suggest that there are two nuclear localizing domains of Daxx.

We also analyzed the association of these Daxx domains with PODs. As expected, Daxx full-length forms POD speckles at low protein levels, while Daxx 555–740 C-terminal domain form occasional PODs. In contrast, Daxx 216–502 and 298–622 fragments do not form POD structures. Interestingly, the aa 1–125 and 1–267 fragments form clear POD structures that colocalize with PML nuclear bodies (Fig. 1C and data not shown). These data suggest that, in addition to the C-terminal PML-interacting domain, Daxx may contain another POD localizing domain at the N-terminal.

Daxx Contains Two Distinct Nuclear Localization Signals

Two consensus nuclear localization sequences of Daxx have been previously predicted [Pluta et al., 1998]; however, prior studies were unable to confirm their function [Pluta et al., 1998; Torii et al., 1999; Jang et al., 2002]. Specifically, replacement of the first predicted NLS at residues 391–395 from RKKRR to RTKSR did not affect nuclear localization of the full-length Daxx [Torii et al., 1999]. Deletion of the C-terminal domain also did not affect Daxx nuclear localization [Pluta et al., 1998; Torii et al., 1999]. A recent study further showed that replacement of the second predicted NLS at residues 627–637 from PPCKKSRKEKK to PPCAASRKEKK or PPCKKSRAEAA also had no effect on Daxx nuclear localization [Jang et al., 2002]. Based on these reports, we reasoned that there are multiple redundant NLSs in Daxx and that mutation of a single NLS is not be sufficient to prevent nuclear localization of the full-length protein.

To identify NLS sequences responsible for nuclear localization Daxx N-terminal domain, we randomly mutated three stretches of basic aa clusters within Daxx 1–267 domain. Specifically, residues 43–60 were mutated from EPHGARGSSSSGGKKCYK to GPMRLRGSSSSGGNICYE (m56) and residues 140–142 were mutated from KKK to NQE (m140). We found that these mutations, alone or in combination, had no effect on nuclear localization of the Daxx N-terminal domain (data not shown). Intriguingly, when the third basic aa cluster at residues 227–231 was mutated from RLKRK to GLEGE (m227), nuclear localization of the Daxx 1–267 domain was completely abolished (Fig. 2A,B). These results suggest that residues 227–231 constitute a functional NLS referred to hereafter as NLS1.

We then investigated putative NLS sequences within the central Daxx 216–502 domain. The predicted NLS at residues 391–395 was mutated from RKKRR to SEETT (m392) and found no effect on nuclear localization (Fig. 2A,B). Intriguingly, introduction of the m227 mutation into aa 216–502 fragment completely abolished its nuclear localization. This result confirms that NLS1 at residues 227–231 is also responsible for nuclear localization of the 216–502 fragment, while the aa 391–395 motif is not a functional NLS. The function of the second predicted NLS at residues 627–637 was then investigated. To bypass NLS1, we replaced the aa 627–637 sequence from PPCKKSRKEKK to PPCNESREEKK (m630A) and PPCNESGEEEE (m630B) in the context of the Daxx aa 555–740 C-terminal domain. Surprisingly, both m630A and m630B mutations completely abolished nuclear localization of this domain (Fig. 2A,B). These results suggest that aa 627–637 motif is indeed a functional NLS, and is referred to as NLS2 hereafter.

NLS1 and NLS2 Determine Nuclear Localization of Full-Length Daxx

To understand the relative contributions of NLS1 and NLS2 to nuclear localization of the full-length Daxx protein, we mutated the NLS motifs in the context of EGFP-full-length Daxx fusion (EGFP-DaxxF) and analyzed their localizations (Fig. 3). As expected, EGFP-DaxxF localizes in the nucleus and forms POD structures. The m140 and m392 mutations have no effects on nuclear localization of EGFP-DaxxF (Fig. 3A). Intriguingly, the m227 mutant shows increased cytoplasmic staining, suggesting that NLS1 indeed contributes to nuclear localization of the full-length Daxx protein. Remarkably, the m630A and m630B mutations cause nearly complete cytoplasmic localization of EGFP-DaxxF, suggesting that NLS2 is the major determinant for nuclear localization of the full-length Daxx. Interestingly, both NLS2 mutants show prominent POD structures in the nucleus, suggesting that a portion of the mutant

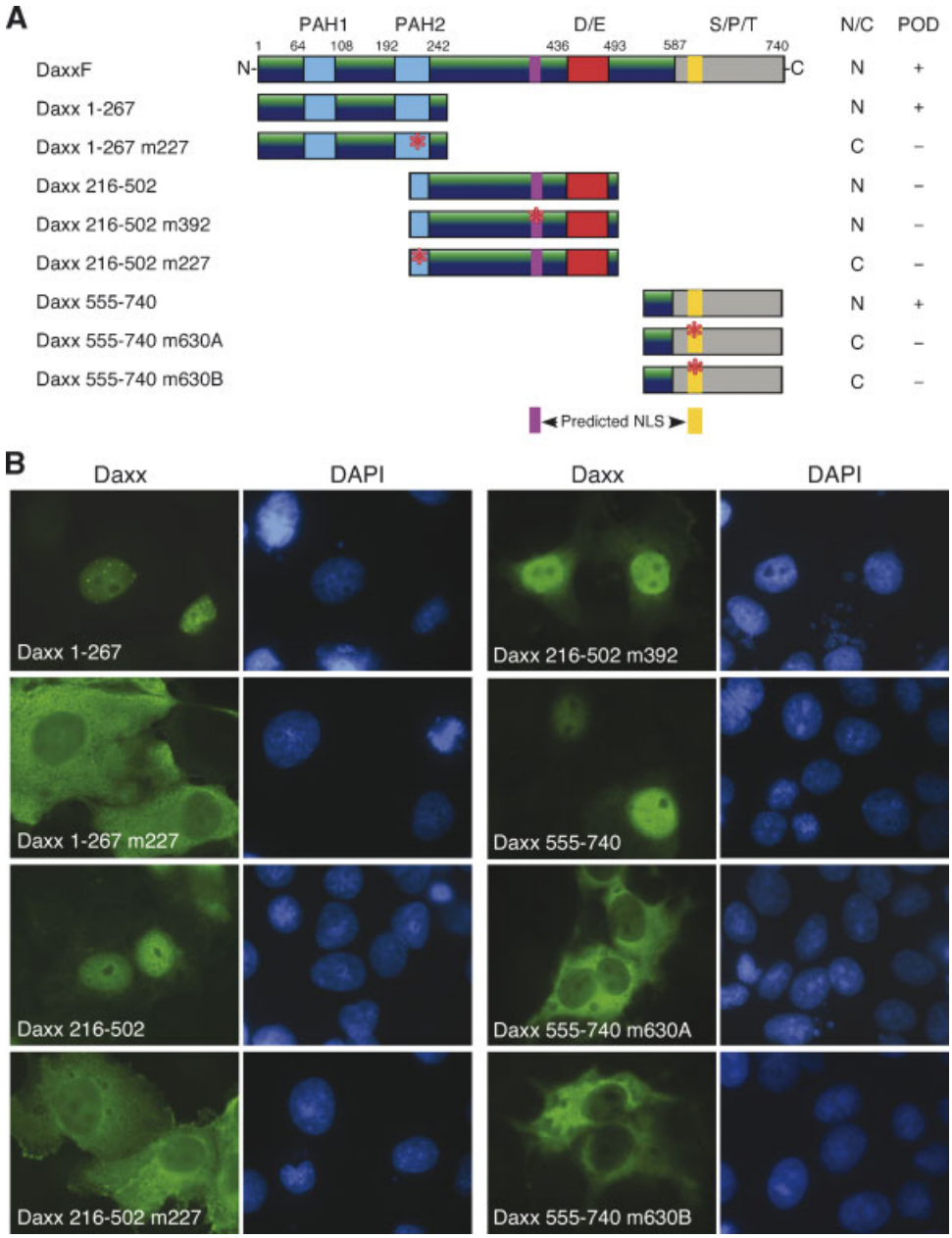

Fig. 2. Site-directed mutagenesis on Daxx nuclear domains unveils two nuclear localization signals. **A**: Schematic representation of three Daxx nuclear domains and site-directed mutations of potential NLSs. The positions of respective mutations are marked by ''['' . The nucleocytoplasmic distribution and POD association are summarized at the right. **B**: Immunofluorescence analyses of the Daxx mutants. COS-7 cells were transfected with indicated constructs, and analyzed with anti-HA antibody followed by FITC-conjugated secondary antibody. DAPI staining shows the nucleus. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteins may still enter into the nucleus and accumulate at PODs.

To completely abolish nuclear localization, we generated double mutants including m140/m630A and m227/m630A in EGFP-DaxxF. As expected, the m140/m630A double mutant displays a similar phenotype as the m630A single mutant because the m140 mutation has no effect on nuclear localization. Intriguingly, the m227/m630A double mutant shows a complete loss of nuclear localization without any detectable POD structures in the nucleus. To confirm this observation, we introduced the m227/m630A double mutation into HA-DaxxF, and found that this double mutant is also localized exclusively in the cytoplasm without forming

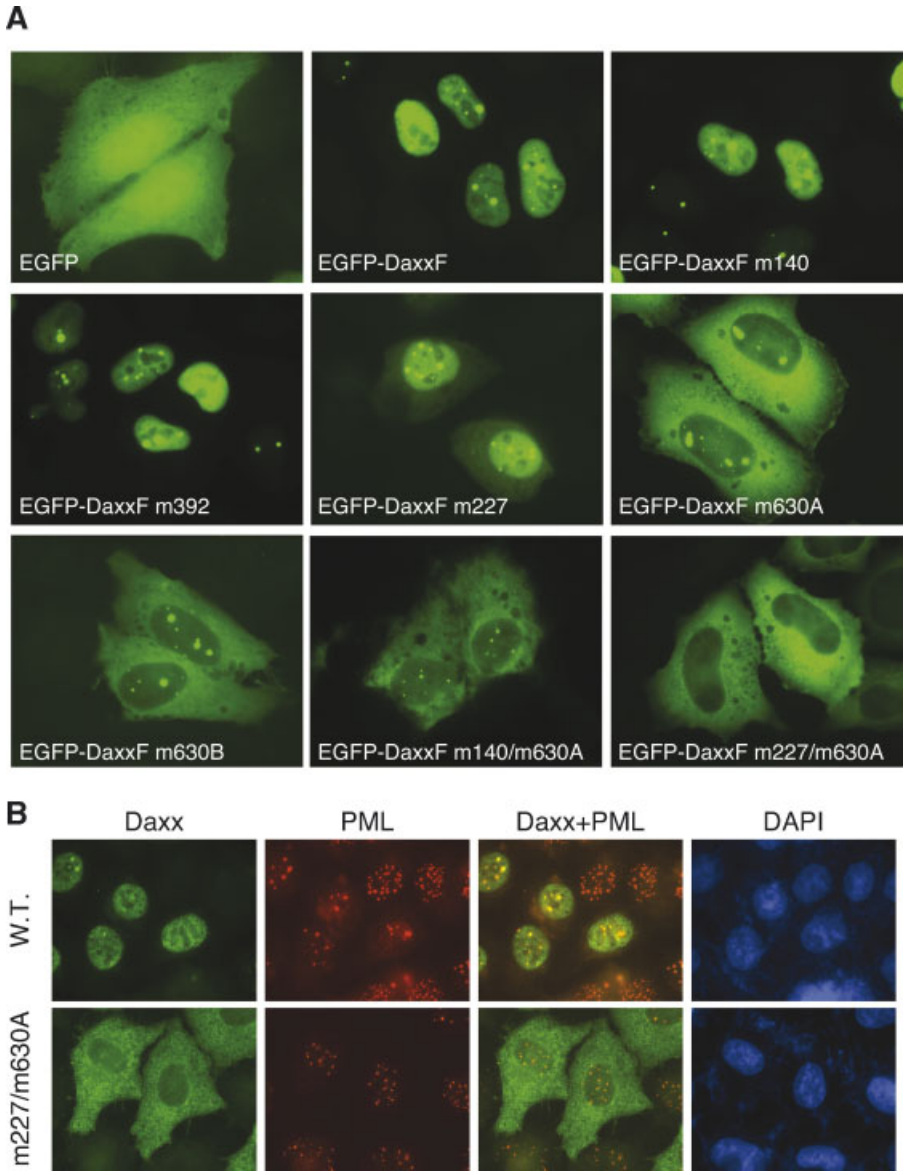

Fig. 3. Site-directed mutagenesis on full-length Daxx reveals the relative involvement of NLS sequences in nuclear localization. **A**: Localization of EGFP-full-length Daxx (EGFP-DaxxF) and its NLS site-directed mutants. Indicated constructs were transiently transfected in HEp2 cells. Transfected cells were fixed with 4% paraformaldehyde and analyzed by fluorescence microscopy. **B**: Localization of HA-DaxxF NLS mutant. HeLa cells were transfected with indicated HA-DaxxF wild type and NLS double mutants (m227/m630A) and analyzed by immunofluorescence staining with anti-HA polyclonal antibody (green) and PML monoclonal antibody 5E10 (red). Cell nuclei were revealed with DAPI staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

any POD structures in the nucleus (Fig. 3B). Therefore, we have created a Daxx mutant that localizes exclusively in the cytoplasm by simultaneously disrupting two independent NLSs.

Both NLS1 and NLS2 Act Autonomously to Mediate Nuclear Import

To further investigate the mechanisms by which Daxx is translocated into the nucleus, we tested whether the NLS1 and NLS2 sequences of Daxx could act autonomously to mediate nuclear import. EGFP-Daxx NLS1 and NLS2 fusion proteins were constructed by fusing Daxx aa 218–235 and 624–740 to EGFP, respectively (Fig. 4A). Interestingly, both EGFP-NLS1 and EGFP-NLS2 localize almost exclusively in the nucleus (Fig. 4B), suggesting that the Daxx NLS1 and NLS2 sequences may act autonomously to mediate nuclear import of heterologous protein.

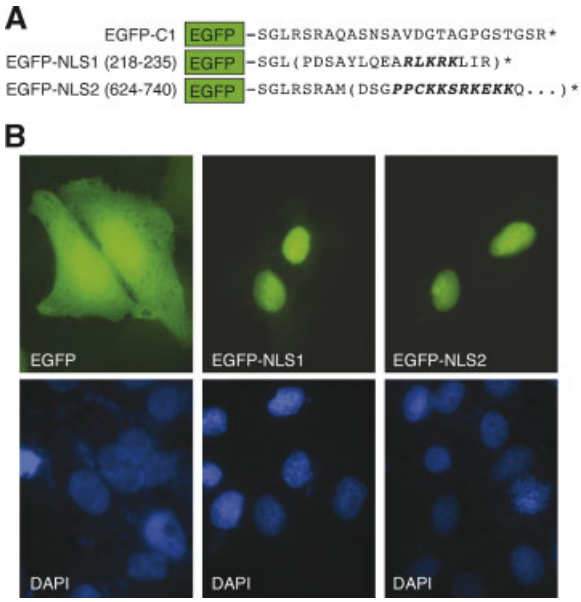

Fig. 4. Daxx NLS sequences act autonomously. **A**: Schematic representation of EGFP-C1, EGFP-Daxx NLS1 (218–235), and EGFP-Daxx NLS2 (624–740) constructs. The Daxx sequences are shown in parentheses with the core NLS sequences in bold and italicized. **B**: Distribution of EGFP-Daxx NLSs fusions. COS-7 cells were transfected with the indicated constructs and analyzed by immunofluorescence microscopy. Cell nuclei were revealed with DAPI staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Daxx Interacts With Importin α3

Several importins are known to directly interact with different cargo proteins in the cytoplasm and to carry the cargos through nuclear pores into the nucleus. To decipher the mechanisms of Daxx nuclear translocation, we analyzed the interactions of Daxx with various importins. A series of GST-importins were tested for interactions with in vitro translated [^{35}S]HA-DaxxF in GST pull-down assays (Fig. 5). Interestingly, full-length Daxx interacts significantly with GST-importin α3, but not with other importins (Fig. 5A). Two smaller Daxx polypeptides display non-specific bindings to all importins, indicating that these importins are capable of protein interaction. The partially degraded importin α4 shows strong binding to p53 (data not shown), indicating that it is able to interact with cargo protein specifically. In addition, we also confirmed the Daxx/importin interaction in a reverse GST pull-down (data not shown). These data suggest that Daxx interacts selectively with importin α3.

We then determined whether binding of importin α3 correlates with nuclear localization of Daxx domains. Indeed, GST-importin α3 interacts with all three Daxx nuclear domains: aa 1–267 (Fig. 5B), aa 216–502 (Fig. 5C), and aa 555–740 (Fig. 5D), but not with the Daxx 298–622 cytoplasmic domain (Fig. 5C), suggesting that nuclear localization of Daxx domains correlate with their abilities to interact with importin α3. To substantiate the role of importin α3 in nuclear translocation of Daxx, we further tested the involvements of Daxx NLS1 and NLS2 in interaction with importin α3. We found that the m227 mutation disrupts the interaction of Daxx 1–267 domain with importin α3 (Fig. 5B), suggesting that NLS1 is involved in interaction with importin α3. Similarly, the m630A mutation also abolishes the interaction of Daxx 555–740 domain with importin α3 (Fig. 5D), suggesting that NLS2 is also involved in interaction with importin α3. These results indicate Daxx uses both NLS1 and NLS2 for direct interaction with importin α3.

Next, we tested the relative contribution of Daxx NLS1 and NLS2 to the interaction between full-length Daxx and importin α3. Intriguingly, m227 alone has only a minor effect on interaction between full-length Daxx and importin α3, consistent with its partial effect on nuclear localization (Fig. 3). Intriguingly, the m630A mutation has a much stronger effect on importin α3 interaction (Fig. 6A). Remarkably, simultaneous disruption of NLS1 and NLS2 by the m630A/m227 double mutation abolishes importin α3 interaction completely. These results strongly indicate that both NLS1 and NLS2 contribute to Daxx/importin α3 interaction, with NLS2 as the major binding site for importin α3.

Importin α molecule contains two NLS-binding sites that directly recognize NLS sequences of cargo proteins. The primary NLS binding site is located at the N-terminal armadillo (arm) repeats 2–4, while the secondary NLS binding site is located at the C-terminal arm repeats 7–9 [Conti et al., 1998; Fontes et al., 2000; Melen et al., 2003; Fagerlund et al., 2005]. If importin α3 indeed mediates Daxx nuclear import, we reasoned that it must utilize the NLS-binding sites to interact with Daxx. To test this hypothesis, we analyzed the interactions of Daxx with arm repeat mutants of importin α3. The arm 3 mutant containing W191A and N195A point mutations disrupts the primary

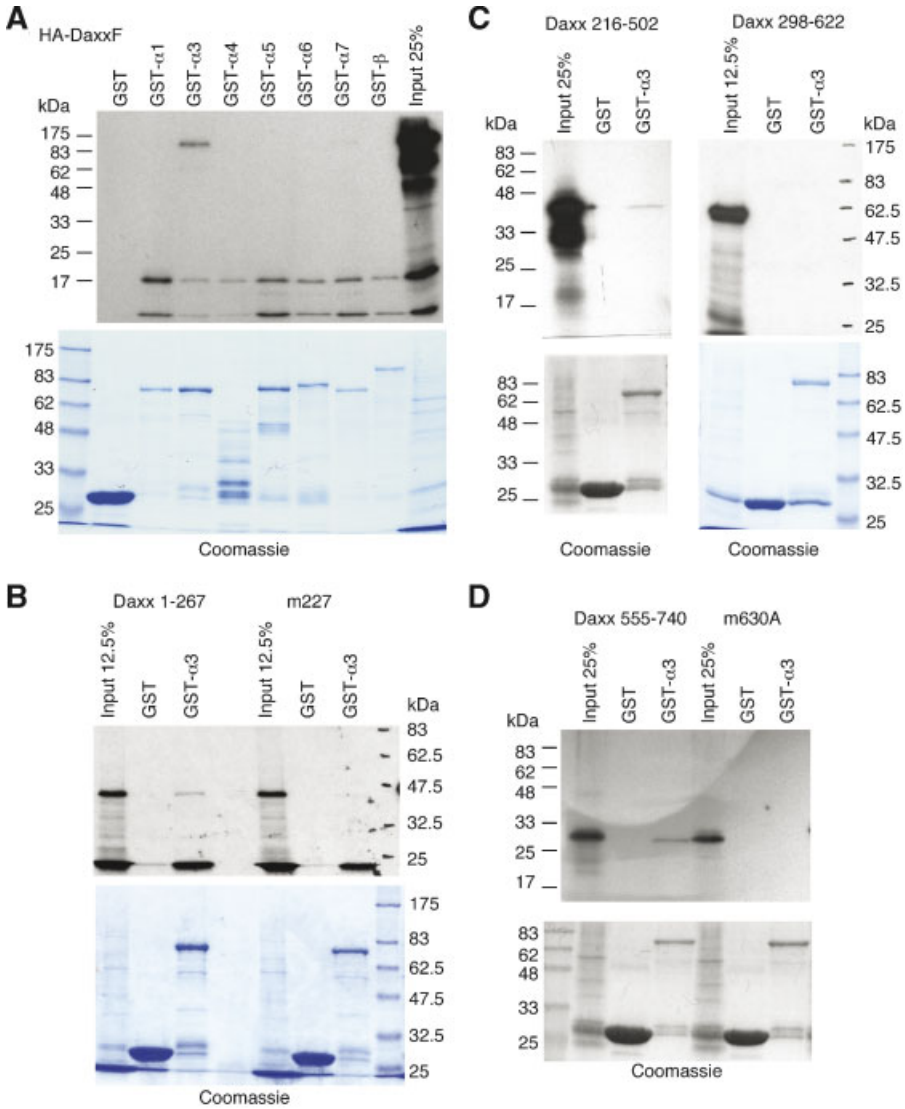

Fig. 5. Daxx interacts with importin α3. **A**: GST pull-down assay showing binding of [^{35}S]HA-DaxxF to GST-importin α3 (GST-α3). HA-DaxxF was produced in reticulocyte lysate and labeled with [^{35}S]methionine. GST-importins were expressed and purified from BL21 cells. Coomassie blue stain shows the relative amount of the GST proteins used in the pull down assay. **B**: GST pull-down assays showing interaction of Daxx 1–267 domain to GST-α3, but not Daxx 1–267 m227 mutant. **C**: GST pull-down assay showing interaction of Daxx 216–502, but not Daxx 298–622 with GST-α3. **D**: GST pull-down assay showing binding of Daxx 555–740 domain to GST-α3 and that m630A mutation abolishes this binding. Coomassie staining of the GST pull-down gels are shown below each autoradiograph. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NLS-binding site [Melen et al., 2003], while the arm 7/8 mutant containing W348A, N352A, W390A, and N394A mutations disrupts the second NLS-binding surface [Fagerlund et al., 2005]. When these importin α3 mutants were tested for Daxx interaction, we found that both mutants fail to interact with Daxx (Fig. 6B). These results suggest that importin α3 indeed uses it NLS-binding sites for interaction with Daxx.

Nuclear Localization is Essential for Transcriptional Function of Daxx

Daxx is known to affect transcription of several transcription factors including GR [Lin et al., 2003; Obradovic et al., 2004; Muromoto et al., 2004a,b] and p53 [Kim et al., 2003; Gostissa et al., 2004; Zhao et al., 2004]. To determine whether nuclear localization is essential for transcriptional function of

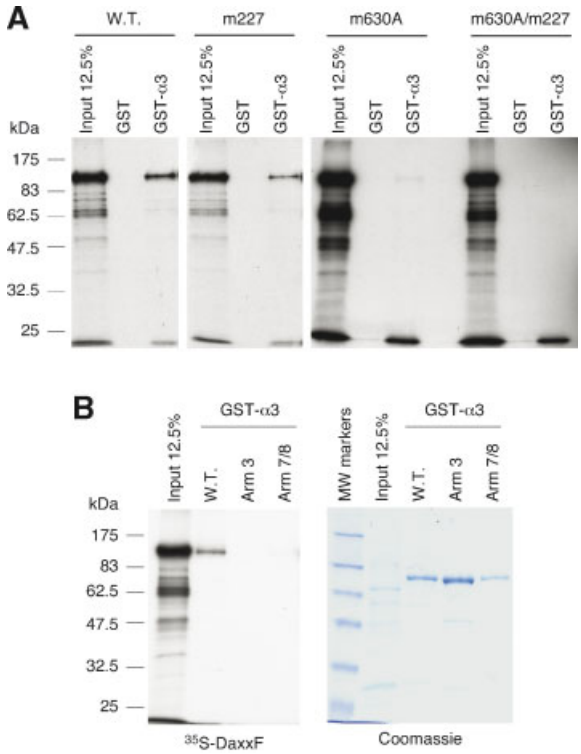

Fig. 6. Requirement of NLS and NLS-binding sites in Daxx/importin α3 interaction. **A**: GST pull-down assay showing progressive disruption of importin α3 bindings to HA-DaxxF mutants by NLS mutations. The m227 (NLS1) mutation has a weaker effect on importin a3 interaction, while the m630A (NLS2) mutation has a stronger effect. The m227/m630A double mutant shows no binding to importin α3. **B**: GST pull-down assay showing that importin α3 arm 3 and arm 7/8 mutants fail to interact with Daxx. The wild type GST-a3 and its arm3 and arm 7/8 mutants were expressed and purified from BL21 cells. The purified proteins are shown by Coomassie blue stain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

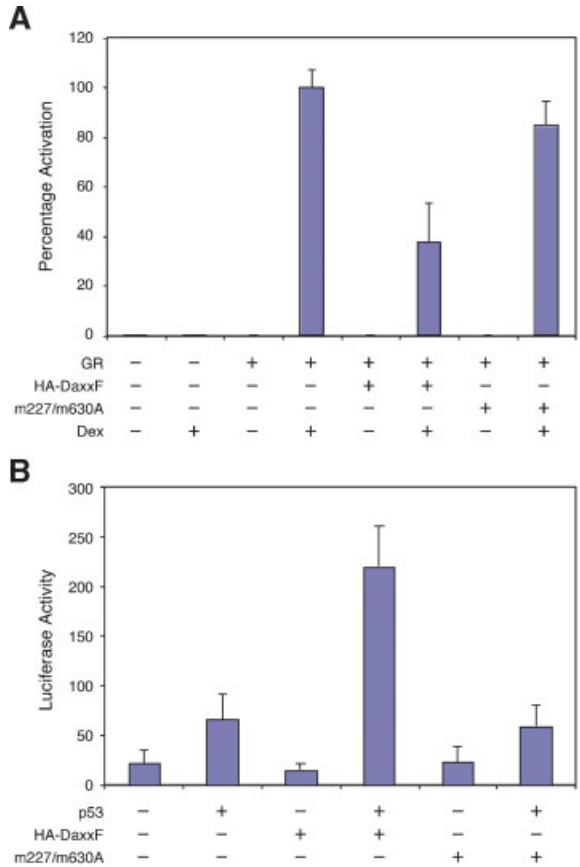

Fig. 7. Nuclear localization is essential for transcriptional function of Daxx. **A**: HA-DaxxF m227/m630A double mutant fails to inhibit GR-mediated transcriptional activation. COS-7 cells were transiently transfected with indicated expression vectors for GR, HA-DaxxF or HA-DaxxF m227/m630A double mutant, together with the MMTV-Luc reporter and a control β-galactosidase expression vector. Average relative luciferase activities were derived from three independent transfections. Where indicated, dexamethasone (Dex) was added at 1 μM for approximately 36 h before luciferase and β-galactosidase activity assays. **B**: HA-DaxxF m227/m630A mutant fails to enhance p53-mediated transcriptional activation. COS-7 cells were transiently transfected with indicated expression vectors for p53, HA-DaxxF or HA-DaxxF m227/m630A double mutant, together with the PG13-Luc reporter and a control β-galactosidase expression vector. Average relative luciferase activities were derived from three independent transfections.

Daxx, we compared the wild-type and m227/m630A double mutant on GR-mediated transcriptional activation on MMTV-luciferase reporter (Fig. 7A). We found that overexpression of Daxx led to approximately 65% reduction of dexamethasone-induced GR-mediated transcriptional activation of the MMTV reporter, consistent with prior studies [Lin et al., 2003; Obradovic et al., 2004; Muromoto et al., 2004a,b]. In contrast, the m227/m630A mutant lost the majority of this inhibitory activity. Both the wild-type and mutant proteins have little effect on basal MMTV promoter activity in the absence of ligand. These results suggest that nuclear localization of Daxx is critical for its transcriptional inhibitory effect on GR.

While Daxx is known to repress transcription in most cases, it may also enhance transcription under certain circumstances. For instance, overexpression of Daxx has been shown to stimulate p53-mediated activation of the pro-apoptotic gene PUMA [Gostissa et al., 2004], as well as Pax5-mediated transcriptional activation in certain cell types [Emelyanov et al., 2002]. We tested the effects of Daxx on p53-mediated transcriptional activation of a p53 luciferase reporter, PG13-luciferase [el-Deiry et al., 1993; Jin et al., 2000]. Overexpression of

Daxx enhances p53-mediated transcriptional activation of the PG13 reporter by ~3-fold (Fig. 7B). Intriguingly, the Daxx m227/m630A double mutant lost this transcriptional coactivator function completely. These results suggest that nuclear localization of Daxx is also essential for its transcriptional coactivator function on p53.

DISCUSSION

In this study, we have identified two NLSs and importin α3 as being responsible for nuclear translocation of the apoptotic and transcriptional regulator Daxx. The aa sequence RLKRK at residues 227–231 of Daxx constitutes the first NLS. This NLS1 is responsible for nuclear localization of the Daxx N-terminal domain. The aa sequence PPCKKSRKEKK at residues 627–637 constitutes the second and major NLS (NLS2). Several putative NLS sequences have been excluded, including a predicted NLS sequence RKKRR at residues 391–395. We demonstrate that simultaneous disruption of NLS1 and NLS2 is essential to abolish nuclear localization of the full-length Daxx, and that both NLS1 and NLS2 can act autonomously to mediate nuclear import of heterologous protein. Furthermore, we identify importin α3 as a Daxx-interacting protein, and that Daxx utilizes NLS1 and NLS2 for interaction with importin α3. Conversely, importin α3 uses its two NLS-binding sites for interaction with Daxx. Finally, we show that nuclear localization of Daxx is essential for its transcriptional regulatory function.

Previously, Torii et al. [1999] analyzed the predicted NLS at residues 391–395 of Daxx and found it not important for nuclear import of the full-length Daxx. In support of this finding, our m392 mutation also has no effect on nuclear localization of the full-length Daxx. Furthermore, when the m392 mutation was introduced into the central 216–502 fragment, no effect on nuclear localization was observed. Intriguingly, when residues 227–231 were mutated in the same domain, nuclear localization is completely lost (Fig. 2). Furthermore, this sequence can act autonomously to mediate nuclear import of heterologous protein (Fig. 4). Consistently, Daxx 298–622 fragment is cytoplasmic as it does not contain NLS1 sequence at residues 227–231. Taken together, we conclude that the 391–395 motif does not function as a NLS, while residues 227–231 constitute a *bona fide* NLS responsible for nuclear localization of Daxx N-terminal domain. The NLS1 is located within the PAH2 domain on the hydrophilic surface of helix B [Hollenbach et al., 1999], suggesting that it may be accessible for interaction with other proteins. The m227 mutation does not alter the amphipathic nature of the helix, and likely would not affect intramolecular interaction between the two helices of the PAH2 domain. PAH repeats are found in several nuclear corepressors including the yeast and mammalian Sin3, and the repeat is distantly related to helix-loop-helix motif. Analogous to the frequent localization of NLSs within DNA binding domains [LaCasse and Lefebvre, 1995], the finding of NLS1 within PAH2 domain of Daxx may have implications for the function of PAH-containing proteins in the nucleus.

The second NLS of Daxx is located in the C-terminal domain at residues 627–637. Previous studies showed that deletion of this sequence did not affect nuclear localization of the full-length Daxx [Pluta et al., 1998; Torii et al., 1999]. A recent study directly tested this NLS by creating K630A/K631A and K634A/K636A/K637A mutations and found no effect on nuclear localization either [Jang et al., 2002]. Paradoxically, our data show that the m630A and m630B mutations at the same motif in three different Daxx constructs drastically affect their nuclear localization (Figs. 2 and 3). Our data indicate that the NLS2 motif actually plays a crucial role in mediating nuclear import of Daxx. To ratify this difference, we have directly tested the same K630A/K631A mutation in our system, and found that this mutation actually impairs nuclear translocation of the full-length Daxx similar to the m630A and m630B mutations (data not shown). The discrepancy might be due to such factors as different cell types and/or experimental conditions, as the prior study co-expressed PML with their mutants in a different cell type, where the overexpressed PML might bring the Daxx mutants into the nucleus and PODs. Since our NLS2 mutation completely blocks nuclear translocation of the Daxx C-terminal domain (Fig. 2), we suggest that NLS2 is the only functional NLS within the C-terminal region of Daxx. Interestingly, disruption of NLS2 alone is not able to completely eliminate nuclear accumulation of the full-length Daxx (Fig. 3A), suggesting that NLS1 is also important for nuclear localization of the

full-length protein. Most interestingly, the NLS2 mutant was found to accumulate also at nuclear PODs, indicating that the NLS2 sequence is not directly involved in POD localization. Remarkably, the double NLS1 and NLS2 mutant is totally cytoplasmic (Fig. 3). These data led us to conclude that Daxx contains two functional NLSs and that these NLSs are not directly involved in POD localization; instead, complete blockage of nuclear localization inevitably prevents localization of Daxx to nuclear PODs.

The mechanism of Daxx nuclear translocation is further elucidated by the revelation that Daxx selectively interacts with importin α3. Among the six human α importins, we identify importin α3 as the only molecule that interacts significantly with Daxx. It is likely that importin α3 physically binds to Daxx in the cytoplasm, and then interacts with importin β at nuclear pores, followed by a Ran-GTP-dependent translocation process through nuclear pores into the nucleoplasm. Our results suggest that importin α3 interacts directly with the NLS1 and NLS2 sequences of Daxx. Importin αs display distinct substrate specificities, as most of them are expressed in the same cells and tissues. The binding affinity and specificity of importin α may be determined by the whole NLS binding groove as well as by sequences flanking the NLS. It has been reported that the SV40 T-antigen NLS peptide binds to all importins [Kohler et al., 1999], whereas the full-length T-antigen binds only to specific importins [Melen et al., 2003]. This may explain why two smaller Daxx polypeptides interact with all importins (Fig. 5A). It is currently unclear why cells utilize only importin α3 instead of all available importins for nuclear import of Daxx.

The discovery of two independent NLSs in Daxx raises the question of how importin α3 interacts with Daxx. Importin α3 contains 10 arm repeats, of which the N-terminal repeats 2–4 are considered the major NLS-binding site, whereas the C-terminal repeats 7–9 form the secondary NLS-binding site. One importin molecule is able to use either the N- or C-terminal sites for recognizing NLS sequences [Melen et al., 2003]. Based on our data, it is reasonable to speculate that one importin α3 may utilize its two NLS-binding sites to recognize NLS1 and NLS2 of Daxx. As NLS2 is the major determinant for nuclear localization of Daxx, we predict that the NLS2 interacts with the N-terminal NLS-binding site of importin α3, whereas NLS1 interacts with the C-terminal NLS binding site. However, the exact configuration of how importin α3 recognizes Daxx will require further investigation.

Finally, we provide strong evidence that nuclear localization of Daxx is essential for its transcriptional regulatory function. We find that overexpression of wild type Daxx inhibits GR-mediated transcriptional activation of the MMTV promoter, consistent with a prior study [Lin et al., 2003]. We further demonstrate that nuclear localization of Daxx is required for this corepressor activity (Fig. 7A). Daxx has been show to interact directly with the AF-1 region of GR through its C-terminal domain [Obradovic et al., 2004]. This C-terminal domain of Daxx also interacts with several other proteins including PML. Interestingly, PML overexpression enhances GR-mediated transcriptional activation in a manner, requiring SUMO-1 modification of PML and sequestration of Daxx to PODs [Lin et al., 2003]. Consistently, we have previously shown that sumoylation of PML is essential for both PML/Daxx interaction and for recruitment of Daxx to PODs, and that sequestration of Daxx to PODs inhibits its transcriptional repressor activity [Li et al., 2000]. Therefore, the fact that a nuclear deficient mutant of Daxx was unable to repress GR activity confirms these findings. Additionally, Daxx has also been implicated in transcriptional coactivation [Emelyanov et al., 2002; Gostissa et al., 2004]. Indeed, we find that overexpression of Daxx stimulates p53 transcriptional activity on the PG13 target promoter (Fig. 7B). Similarly, this coactivator function of Daxx is abrogated by the NLS mutation, suggesting it is also a nuclear event.

Taken together, this study has provided several new insights into the molecular mechanisms governing nuclear localization of Daxx. These data also provide important tools for future investigation of the signaling pathways and function of Daxx.

ACKNOWLEDGMENTS

We are most grateful to Dr. Riku Fagerlund (National Public Health Institute, Finland) who generously provided us the importin constructs. We thank Michael Chisamore for helpful reading of this manuscript. This work was supported by an RO1 grant from National Cancer Institute

and National Institutes of Health to JDC (CA87074).

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