Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek

Andrew D. Hollenbach, Craig J. McPherson*, Edwin J. Mientjes, Rekha Iyengar and Gerard Grosveld‡

1 Department of Genetics, St. Jude Children’s Research Hospital, 332 North Lauderdale, Memphis, TN 38105, USA
*Present address: Department of Infectious Diseases, Parke Davis Pharmaceuticals, 2800 Plymouth Road, Ann Arbor, MI 28105, USA
‡Author for correspondence (e-mail: gerard.grosveld@stjude.org)

Summary
Human Daxx is a protein that functions, in part, as a transcriptional co-repressor through its interaction with a growing number of nuclear, DNA-associated proteins. To determine the mechanism by which hDaxx represses transcription, we used conventional chromatography to isolate endogenous hDaxx. We determined that hDaxx has an apparent molecular weight of 360 kDa, which is consistent with the fact that multiple domains of hDaxx are required for transcriptional repression and suggests that hDaxx associates with multiple proteins. Using co-fractionation and co-immunoprecipitation we demonstrate that hDaxx associates with proteins that are critical for transcriptional repression, such as histone deacetylase II, constituents of chromatin such as core histones H2A, H2B, H3 and H4, and Dek, a chromatin-associated protein reported to change the topology of DNA in chromatin in vitro. We also demonstrate a requirement for the SPT domain and the first paired amphipathic helix of hDaxx for its association with histone deacetylase II and acetylated histone H4, respectively. Finally, we provide evidence suggesting that the association of hDaxx with chromatin-related proteins is dependent on the post-translational phosphorylation status of hDaxx. A working model for the repressive action of hDaxx through its association with chromatin related proteins is presented.

Key words: Daxx, HDAC II, Histone, Dek

Introduction
Daxx is a ubiquitously expressed protein (Hollenbach et al., 1999; Kiriakidou et al., 1997) that was originally isolated from a yeast two-hybrid screen aimed at identifying proteins that interact with the cytoplasmic tail of the Fas receptor (FasR), the region of the receptor known as the ‘death domain’. It was originally proposed that hDaxx promoted apoptosis, as overexpression of hDaxx was observed to enhance Fas-dependent apoptosis (Yang et al., 1997). However, in contrast to its proposed role in promoting apoptosis, Daxx-null mice were embryonic lethal by day 9.5 of gestation and exhibited extensive apoptosis (Michaelson et al., 1999). This observation is contradictory to a role for hDaxx in Fas-dependent apoptosis and argues against a role for Daxx in promoting Fas-dependent cell death. The Daxx knockout embryos also exhibited a highly disorganized physiology (Michaelson et al., 1999), suggesting that Daxx is essential for mouse development.

In addition to the originally proposed role for hDaxx in promoting apoptosis, it was also suggested that it acted as a link between FasR and ASK1 (Chang et al., 1998), a downstream signaling kinase involved in Fas-dependent apoptosis. This observation predicted a cytoplasmic cellular localization for hDaxx. However, it has since been demonstrated that hDaxx is a strictly nuclear protein (Hollenbach et al., 1999; Kiriakidou et al., 1997; Pluta et al., 1998), with at least two identified functions in the nucleus. First, hDaxx has been identified as a component of nuclear promyelocytic leukemia protein (PML) oncogenic domains (PODs) (Everett et al., 1999; Zhong et al., 2000). It was demonstrated that the observed sensitivity of cells overexpressing hDaxx to apoptosis is mediated through the nuclear interaction of hDaxx with PODs and not through a direct interaction with the Fas receptor in the cytoplasm (Torii et al., 1999; Villunger et al., 2000; Zhong et al., 2000). Daxx associates with PODs through a direct interaction with PML, a critical component of PODs (Everett et al., 1999; Ishov et al., 1999; Li et al., 2000a). The interaction is a dynamic, cell cycle regulated event and is dependent on the post-translational modification of PML by the small ubiquitin-related modifier SUMO-1 (Ishov et al., 1999; Lehembre et al., 2001; Li et al., 2000a).

In addition to its presence in PODs, we showed that hDaxx could act as a transcriptional co-repressor. Consistent with this role hDaxx contains four structural domains commonly found in transcriptional regulatory proteins: two predicted paired amphipathic helices, an acid-rich domain and a Ser/Pro/Thr (SPT)-rich domain (Hollenbach et al., 1999). The tethering of hDaxx to DNA by fusing it to the GAL4 DNA-binding domain (GAL4-DBD) resulted in an 85% repression of transcriptional activity from the constitutively active thymidine kinase promoter that contained a GAL4-binding site (Hollenbach et al., 1999; Li et al., 2000a). The tethering of hDaxx to DNA by fusing it to the GAL4 DNA-binding domain (GAL4-DBD) resulted in an 85% repression of transcriptional activity from the constitutively active thymidine kinase promoter that contained a GAL4-binding site (Hollenbach et al., 1999; Li et al., 2000a). The binding of hDaxx to DNA by fusing it to the GAL4 DNA-binding domain (GAL4-DBD) resulted in an 85% repression of transcriptional activity from the constitutively active thymidine kinase promoter that contained a GAL4-binding site (Hollenbach et al., 1999; Li et al., 2000a). The binding of hDaxx to DNA by fusing it to the GAL4 DNA-binding domain (GAL4-DBD) resulted in an 85% repression of transcriptional activity from the constitutively active thymidine kinase promoter that contained a GAL4-binding site (Hollenbach et al., 1999; Li et al., 2000a).
hDaxx form was shown to result, in part, from a post-translational phosphorylation (Hollenbach et al., 1999). However, only the non-phosphorylated 70 kDa form of hDaxx interacts with the DNA-bound transcription factor Pax3, which represses the transcriptional activity of Pax3 by nearly 80% (Hollenbach et al., 1999). In a later report, hDaxx was also shown to repress the transcriptional activity of ETS-1, a member of the ets family of transcription factors (Li et al., 2000b), thus supporting the role of hDaxx as a transcriptional co-repressor. To date, the mechanism by which hDaxx exerts its repression activity is poorly understood. Histone deacetylation, which plays a critical role in transcriptional silencing of actively transcribed chromatin by inducing chromatin condensation onto naked DNA (Pazin and Kadonaga, 1997; Wolffe and Hayes, 1999), has been implicated in hDaxx-mediated transcriptional repression (Li et al., 2000a). However, an exact mechanism describing the repression activity of hDaxx has yet to be determined. Therefore, to better understand the mechanism of hDaxx repression, we have created a series of hDaxx deletion mutants fused to the GAL4 DNA-binding domain (DBD). In the present work, we demonstrate that multiple domains of hDaxx exert transcriptional repression, which suggests that hDaxx may associate with multiple proteins. Using standard chromatography and co-immunoprecipitation analyses, we demonstrate that hDaxx associates with the core histones H2A, H2B, H3 and H4; histone deacetylase II (HDAC II), which is critical for transcriptional repression; and Dek, a protein that alters the superhelical density of DNA in vitro (Alexiadis et al., 2000) and associates with chromatin in vivo (Kappes et al., 2001). Finally, consistent with the requirement of multiple domains for the repression activity of hDaxx, we demonstrate that both the SPT domain and the first paired amphipathic helix of hDaxx are necessary for its association with HDAC II and acetylated histone H4, respectively.

Materials and Methods

Construction of hDaxx deletion mutants and protein expression

All deletion mutant constructs were cloned in frame with the GAL4 DBD (amino acids 1-144) in the vector pM2. The N-terminal and C-terminal deletions are identified by the specific amino acids that were removed. The hDaxxΔ574-740 deletion was constructed by removal of the C-terminal HindIII fragment from GAL4-hDaxx. The hDaxxΔ1-573 deletion was constructed by cloning the C-terminal HindIII fragment of hDaxx into pM2. The remaining C- and N-terminal deletions were generated by PCR amplification and standard cloning techniques. The domain-specific and nuclear-localization-specific deletion mutants were constructed by overlap extension PCR (Ho et al., 1989). Primers were complementary to the regions of hDaxx to be deleted and were engineered to utilize unique restriction sites, allowing the efficient cloning of the amplified products as ‘cassettes’. Specifically, the hDaxxΔPAH1 PCR product removed the first predicted paired amphipathic helix (nucleotides 189-324), and the resulting PCR cassette was cloned into the AccI site present in the pM2 polylinker and at position 375 in hDaxx. The hDaxxΔPAH2 PCR product removed the second predicted paired amphipathic helix (nucleotides 574-726), and the resulting PCR cassette was cloned directionally into the hDaxx NgoMI site at position 354 and the hDaxx EagI site at position 748. The hDaxxΔAD PCR product removed the acid-rich domain (nucleotides 1299-1479), and the resulting PCR cassette was cloned into the hDaxx Ksal site at position 1077 and the hDaxx BsrXI site at position 2056. The hDaxxΔSPT PCR product removed the final 495 bp of sequence (nucleotides 1725-stop), and the resulting PCR cassette was cloned into the Ksal site at position 1077 of hDaxx and the XbaI site in the pM2 polylinker. Finally, the ANLS1 PCR product removed the first predicted nuclear localization sequence (nucleotides 1162-1185), and the ANLS2 PCR product removed the second predicted nuclear localization sequence (nucleotides 1888-1902). Both PCR products were cloned into the hDaxx Ksal site at position 1077 and the hDaxx BsrXI site at position 2056. The deletion cassettes were then used to construct the multiple domain deletion mutants. For example, the hDaxxΔPAH1-2 deletion construct was created by cloning the NgoMI-Eagl ΔPAH2 cassette into the NgoMI-Eagl site of hDaxxΔPAH1. All constructs were sequenced to confirm that they are in frame with the GAL4 DBD and that no point mutations were introduced during PCR amplification.

To determine protein expression, NIH3T3 cells (5x10⁴) were plated on 100 mm dishes and transfected the following day with 10 µg of the pM2-hDaxx deletion constructs using the Fugene6 method (Boehringer Mannheim, Germany) according to the manufacturer’s specifications. After 48 hours, the cells were harvested, resuspended in PBS containing both protease and phosphatase inhibitors (0.1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml Aprotinin, 1 mM β-glycerophosphate, 1 mM NaF and 0.1 mM NaVO₄) and lysed by four rounds of freezing and thawing. Cell lysates (30 µg) were resolved by either 15% or 8% SDS-PAGE, blotted to Immobilon-P membrane (Millipore, Bedford, MA), and the protein was detected by western analysis using the mouse anti-GAL4 DBD monoclonal antibody (Santa Cruz Biotechnology, CA).

Immunofluorescence

To determine the cellular localization of the individual deletion mutants, NIH3T3 cells (5x10⁴) were plated on one-chamber polystyrene vessel tissue-culture-treated glass slides (Becton Dickinson, NJ) and transfected the following day with 2 µg of each of the pM2-hDaxx deletion constructs. After 48 hours, the cells were fixed and permeabilized as previously described (Lam et al., 1999) and incubated for 1.5 hours with a 1:2000 dilution of the anti-GAL4 DBD antibody. After extensively washing with PBS, the cells were incubated with FITC-conjugated goat anti-mouse antibody (1:250 dilution) for 45 minutes, washed with PBS and mounted with Vectashield® mounting medium (Vector Laboratories, Burlingame, CA) that contained 3 µM 4’,6-diamindino-2-phenylindole (DAPI, Sigma, MO). Slides were examined using an Olympus BX50 fluorescent scope.

Transcriptional assays

To determine the repression activity of the hDaxx deletion mutants, NIH3T3 cells (3x10⁵) were plated on 60 mm dishes and transfected the following day using the Fugene6 method. The Fugene6-DNA complex consisted of 10 fmol of the indicated pM2-hDaxx deletion constructs, 500 ng of the secreted alkaline phosphatase (SEAP) control plasmid under control of the MAP1 promoter and either 1 µg of the 1X-GAL4-TK-CAT reporter plasmid containing one GAL4-DNA-binding site or 0X-GAL4-TK-CAT reporter plasmid containing no GAL4-DNA-binding sites. This reporter contained the chloramphenicol acetyl transferase (CAT) gene under control of the constitutively active thymidine kinase promoter containing either one or no GAL4-DNA-binding sites. After 48 hours, the medium was assayed for SEAP activity as previously described (Bram et al., 1993), and the cells were harvested and assayed for CAT activity as previously described (Ausubel et al., 1996). The percentage of [14C]chloramphenicol acetylation was quantified from thin-layer chromatography plates using a Molecular Dynamics Phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ). The transfection efficiency was normalized relative to the SEAP activity, and values represent the average and standard deviation from four independent determinations.
Co-fractionation of hDaxx, HDAC II, core histones and Dek
To determine whether hDaxx, HDACII, histones and Dek co-fractionated, U937T cells (1.2×10⁶) (Boer et al., 1998) stably expressing FLAG-epitope tagged Dek were harvested and resuspended in 10 ml of a buffer containing 20 mM HEPES (pH 7.9), 150 mM KCl and all protease and phosphatase inhibitors as described above. The cells were sonicated two times for 20 seconds on power level three with a 550 Sonic Dismembrator (Fisher, Pittsburgh, PA), separated into 10×1 ml aliquots, and each aliquot was sonicated for an additional three rounds. The debris was removed by centrifugation in an Eppendorf Centrifuge 5415D microfuge at 16,100 g for 10 minutes. The supernatant was removed, and glycerol and Tween 20 were added to final concentrations of 20% and 0.1%, respectively (Buffer PLB). The total cell lysate (70 mg) was loaded onto a HiPrep® Sephacryl S-300 size exclusion column (26×600 mm, Pharmacia, Belgium) pre-equilibrated with buffer PLB. Proteins were eluted with a flow rate of 1 ml/minute into 5 ml fractions. The presence of hDaxx was determined by Deoxycholate/Trichloroacetic acid (DOC/TCA) precipitating 20 µl aliquots of each individual fraction followed by acetone precipitation and separating the precipitated proteins on a 4-20% SDS-PAGE gradient gel. The gel was blotted to Immobilon-P membrane (Millipore, Bedford, MA), and hDaxx was detected by western analysis using a rabbit polyclonal antibody described previously (Hollenbach et al., 1999). Fractions were also analyzed for the presence of HDACII using a mouse monoclonal anti-HDACII antibody (Santa Cruz sc-9959, CA), acetylated histone H4 using a rabbit polyclonal anti-acetylated histone H4 antibody (Upstate Biotechnology #06-866, Lake Placid, NY) and Dek-FLAG using a mouse monoclonal anti-FLAG antibody (Sigma, St. Louis, MO). The apparent native molecular weight of endogenous hDaxx was determined from a standard curve of proteins of known molecular weights.

The fractions containing hDaxx were combined, and the protein (20 mg, 3.5-fold purification over crude lysate) was loaded onto a Resource-Q® anion exchange column (Pharmacia, Belgium) 6 ml bed volume) previously equilibrated with buffer PLB. The column was washed with 10 column volumes of buffer PLB (60 ml) at a flow rate of 1 ml/minute into 5 ml fractions, and proteins were eluted with a linear gradient of 0.15-1 M KCl in buffer PLB at the same flow rate. The presence of hDaxx, HDACII, acetylated histone H4 and Dek-FLAG in each fraction was determined as described above. The fractions containing hDaxx were combined, concentrated to 2 ml, and the protein (2 mg, 35-fold purification over crude lysate) was loaded onto a Superdex™ HR-200 gel filtration column (20×320 mm, Pharmacia, Belgium) previously equilibrated with buffer PLB. Proteins were eluted with a flow rate of 1 ml/minute into 2.5 ml fractions, and the presence of all proteins was determined as described above. The apparent native molecular weight of hDaxx was determined from a standard curve of proteins of known molecular weights. Approximately 120 µg of hDaxx and its associated proteins were obtained from 140 mg of total cellular extract, resulting in an approximately 1150-fold purification.

Mass spectral analysis of histones
We determined the presence of histones by mass spectral analysis because commercially available antibodies did not recognize non-acetylated histones by western analysis in our hands. 20 µg of protein from the final fraction of the purification were separated by 4-20% SDS-PAGE, and the proteins were visualized by Coomassie staining. Following electrophoresis and visualization, the gel was dried and the proteins corresponding to the molecular weights of histones H2A, H2B, H3 and H4 were excised from the gel. The protein in the excised gel piece was reduced, alkylated with iodoacetamide and digested with trypsin. Tryptic peptides were extracted and subjected to combined capillary liquid chromatography/tandem mass spectrometry. Mass spectrometry was performed using a ThermoQuest LCQ-DECA ion-trap mass spectrometer with an electrospray ion source. Fragment ions (MS²) spectra were subjected to search of the NCBI non-redundant protein database using the SEQUEST program of Eng and Yates marketed by ThermoQuest.

Co-immunoprecipitation of hDaxx, HDAC II, Dek and acetylated histone H4
To demonstrate a physical association of hDaxx, HDAC II, Dek and acetylated histone H4 and to map the domains of hDaxx responsible for its association with HDACII and acetylated histone H4, co-immunoprecipitation experiments were performed. Because U937T cells did not transfact well by the Fugene6 method, 293T cells were used. 293T cells (1×10⁶) were plated in 100 mm dishes and transfected the following day with 5 µg of the mammalian expression vectors encoding either GAL4-hDaxx, GAL4-hDaxx1-132, GAL4-hDaxx1-352 or GAL4-hDaxx-SPT using the Fugene6 method according to the manufacturer’s specifications. After 48 hours, the cells were lysed on ice in NP40 lysis buffer (0.4 M NaCl, 0.2 mM EGTA, 10% glycerol, 1% NP40 and all protease and phosphatase inhibitors as described above), the cell debris was removed by centrifugation at maximum speed in a Sorvall RMC 1Y microfuge for 15 minutes at 4°C, and the resulting cell extracts were preclreated by incubating them with Gamma Bind plus Sepharose resin (Pharmacia, Belgium) for 2 hours at 4°C. Total cell lysate (150 µg) was incubated with a mouse monoclonal anti-GAL4 DBD antibody (clone RK5C1, Santa Cruz, CA) overnight at 4°C on a rotary shaker; immune complexes were collected with Gamma Bind Plus Sepharose beads; and the beads were washed four times with ice-cold NP40 lysis buffer. The pellets were resuspended in 30 µl of SDS-PAGE buffer and boiled for 5 minutes. Supernatants were resolved by 4-20% gradient SDS-PAGE, blotted to Immobilon-P membrane and either HDAC II or acetylated histone H4 were detected by western analysis using a mouse monoclonal anti-HDAC II or rabbit polyclonal anti-acetylated histone H4 antibody, respectively. Unfortunately, we were unable to analyze the presence of endogenous Dek owing to the non-specific interaction of Dek with the gamma-bind plus resin. To confirm overexpression of all deletion constructs, 20 µg of total cell lysate was resolved by 4-20% SDS-PAGE, blotted to Immobilon-P membrane, and protein was detected by western analysis using the mouse monoclonal anti-GAL4 DBD antibody as described above.

Alternatively, associated proteins were also analyzed by immunoprecipitation of Dek-FLAG. Fractions from the Sephacryl S-300 column that contained hDaxx, Dek, acetylated histone H4 and HDAC II (fractons 9-13) were combined and passed over an anti-FLAG antibody affinity column (Sigma, St. Louis, MO). The column was washed extensively with buffer PLB, and proteins that were retained by the column were competitively eluted with PLB buffer containing 100 µg/ml FLAG peptide. The eluted proteins were DOC/TCA/acetone precipitated, separated by 4-20% SDS-PAGE, transferred to Immobilon-P membrane, and hDaxx II and acetylated histone H4 were detected by western analysis as described above, or the core-histones H2A, H2B, and H3 were visualized by silver stain.

Histone deacetylase activity assays
To determine the ability of GAL4-hDaxx to immunoprecipitate histone deacetylase activity, 293T cells (1×10⁶) were plated in 100 mm dishes and transfected the following day with 5 µg of the mammalian expression vectors encoding either GAL4-hDaxx or GAL4 by the Fugene6 method according to the manufacturer’s specifications. After 48 hours, the cells were lysed with RIPA buffer containing all protease and phosphatase inhibitors and preclreated, as described above. Total cell lysate (100 µg) was immunoprecipitated with the anti-GAL4 antibody, and after extensive washing with lysis buffer, the beads containing the immune complexes were resuspended.
in 200 μl of HDAC assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol and all protease). [3H]Acetyl histone H4 (730,000 cpm/μl), previously prepared according to the manufacturer’s specifications (Upstate Biotechnology, Lake Placid, NY), was added to the immune complexes, and the reactions were incubated for 36 hours at room temperature. The reactions were stopped by the addition of 50 μl of quenching solution (1.0 M HCl, 160 mM acetic acid), and the released [3H]acetate was extracted and quantified according to the manufacturer’s specifications (Upstate Biotechnology, Lake Placid, NY). As a positive control for the histone deacetylase reaction, 10 μg of HeLa nuclear extract was incubated with the HDAC assay buffer and [3H]acetate was analyzed as described above. The specificity of the histone deacetylase reaction was determined by parallel reactions that were incubated with 50 mM sodium butyrate, a specific histone deacetylase inhibitor.

Results
GAL4-hDaxx deletion constructs
To understand the mechanism by which hDaxx exerts its transcriptional repression activity, we created a series of deletion mutants that target the four identified structural domains of hDaxx. All of the deletion constructs were fused in frame to the GAL4-DNA-binding domain (DBD) (Fig. 1). These mutants included successive C-terminal (hDaxxΔ574-740, hDaxxΔ334-740, and hDaxxΔ157-740) and N-terminal (hDaxxΔ1-132, hDaxxΔ1-352, and hDaxxΔ1-573) deletions, which removed regions surrounding the identified structural domains of hDaxx. We also included the C-terminal deletion hDaxxΔ626-740 that was previously described to be a constitutively active mutant in its putative role in Fasdependent apoptosis (Yang et al., 1997). In addition, we constructed two mutants that retain only the regions surrounding the paired amphipathic helix number two (hDaxxΔ133-333) and the acidic domain (hDaxxΔ353-573). Finally, we generated a series of small deletion mutants that include the removal of each individual domain (ΔPAH1, ΔPAH2, ΔAD, and ΔSPT), the removal of two domains (ΔPAH1-2, ΔPAH1-AD, ΔPAH1-SPT, ΔPAH2-AD, ΔPAH2-SPT, and ΔAD-SPT), the removal of three domains (ΔPAH1-2-AD, ΔPAH1-2-SPT, ΔPAH1-AD-SPT, and ΔPAH2-AD-SPT) and the removal of all four domains of hDaxx (ΔPAH1-2-AD-SPT).

Transcriptional repression activity of GAL4-hDaxx deletion mutants
Before we could use the deletion constructs in transcriptional repression assays, several controls needed to be performed. First, to demonstrate that all deletion mutants were expressed at similar levels, each of the constructs was individually transfected into NIH3T3 fibroblasts, and the presence of mutant protein was detected by western analysis using a mouse anti-GAL4 DBD monoclonal antibody. The majority of the deletion constructs expressed similar levels of protein (Fig. 1) except for Δ1-352, which in addition to expressing protein of the correct molecular weight also demonstrated a considerable amount of protein degradation (Fig. 2B). Interestingly, we noted barely detectable levels of protein with the majority of constructs in which the acid-rich domain had been removed (ΔAD, ΔPAH1-AD, ΔAD-SPT, ΔPAH1-2-AD, ΔPAH1-AD-SPT, ΔPAH1-2-AD-SPT, hDaxxΔ334-740, and hDaxxΔ133-333) (Fig. 1). In addition, the deletion constructs that lacked the acid-rich domain but still retained the N-terminal paired amphipathic helix (ΔPAH2-AD, ΔPAH2-AD-SPT) expressed protein of the correct molecular weight at a lower level than full-length hDaxx (Fig. 1). A pulse-chase analysis of select deletion mutants confirmed that the proteins were being expressed and that the observed reduction in the steady-state level of protein expression was caused by a decrease in the half-life of deletion mutants lacking the acid-rich domain. The specific removal of the acid-rich domain decreased the normal stability of GAL4-hDaxx by greater than four-fold (data not shown), suggesting that the acid-rich domain is essential for the stable expression of hDaxx.

Second, hDaxx contains two predicted nuclear localization signals (NLS): one present in the SPT domain (NLS2, K630KSRK634) and one immediately N-terminal to the acid-rich domain (NLS1, R391KKRR395) (Kiriakidou et al., 1997; Li et al., 2000a). Therefore, to confirm that the hDaxx deletion mutants maintained a nuclear localization, we transiently transfected NIH3T3 cells with each individual construct, and the localization of the protein was visualized by immunofluorescence using the anti-GAL4 DBD monoclonal antibody. Consistent with previous reports (Everett et al., 1999; Hollenbach et al., 1999; Ishov et al., 1999; Kiriakidou et al., 1997; Pluta et al., 1998), GAL4-hDaxx demonstrated a strict nuclear staining (Fig. 1). In addition, nuclear staining was also observed for each of the deletion mutants containing an intact SPT domain (Fig. 1). By contrast, despite the presence of a predicted NLS in hDaxx immediately N-terminal to the acid-rich domain, hDaxx deletion mutants missing all or part of the SPT domain demonstrated a diffuse staining throughout the cell (Fig. 1). The loss of strict nuclear staining upon removal of the SPT domain indicated that NLS2 is critical for the nuclear localization of hDaxx. Consistent with this observation, we found that the specific deletion of NLS2 was sufficient to remove the strict nuclear localization of GAL4-hDaxx. However, the removal of NLS1 had no effect on the nuclear localization of GAL4-hDaxx (data not shown). This observation provided direct evidence that the NLS2 is necessary and sufficient for the localization of hDaxx to the nucleus.

Finally, the deletion constructs that localized strictly to the nucleus and produced stable protein (hDaxx, hDaxxΔ1-573, hDaxxΔ1-132, ΔPAH1-2, ΔPAH2-AD, hDaxxΔ1-352, ΔPAH2, and ΔPAH1) were tested for their ability to repress transcription. Deletion mutants that expressed stable protein but displayed a diffuse cellular localization were cloned in frame with the SV40 NLS between the GAL4 DBD and the hDaxx mutant construct [(NLS)ΔPAH1-SPT, (NLS)SAPT, (NLS)ΔPAH2-SPT, (NLS)ΔPAH1-2-SPT, and (NLS)hDaxxΔ353-573]. The presence of the SV40 NLS did not affect protein stability (Fig. 2B) nor did it affect the transcriptional repression activity of GAL4-hDaxx (data not shown). It was, however, sufficient to restore the strict nuclear localization of the deletion mutants (data not shown). Consistent with previous reports (Hollenbach et al., 1999; Li et al., 2000a), expression of full-length GAL4-hDaxx resulted in an 85% reduction of transcriptional activity (Fig. 2A). This reduction was dependent on GAL4-hDaxx binding to DNA and was not caused by titration of other essential factors by hDaxx as minimal repression (≤15%) was observed when either the GAL4 DBD or the GAL4-DNA-binding sites were
removed (data not shown) (Hollenbach et al., 1999). In the same manner as with full-length GAL4-hDaxx, the presence of the SPT domain in the deletion mutants was sufficient to provide an 85% reduction of transcriptional activity (Fig. 2A). However, the SPT domain is not solely responsible for the repression activity of hDaxx, as the construct that had the SPT domain deleted ((NLS)ΔSPT) retained transcriptional repression activity similar to that of wild-type hDaxx (Fig. 2A). Removal of PAH1 (hDaxxΔ1-132 and ΔPAH1) or PAH2 (ΔPAH2) independently was insufficient to alter the repression activity of hDaxx (Fig. 2A). Analysis of the transcriptional repression activity of several of the combined deletion mutants [(NLS)ΔPAH2-SPT and (NLS)ΔPAH1-2-SPT] or the deletion mutant that retained only the region surrounding the acid-rich domain [(NLS)hDaxx353-573] demonstrated that although there was a significant increase in transcriptional activity relative to full-length hDaxx (Fig. 2A), these constructs still retained transcriptional repression activity. The observed differences in activity were not caused by different levels of protein expression as all deletion constructs expressed protein to similar levels, with only Δ1-352 showing any considerable degradation (Fig. 2B). Overall, therefore, the results of this deletion analysis indicate that multiple domains of hDaxx are required for transcriptional repression.

Co-fractionation of hDaxx, Dek, HDAC II and core histones

Our data suggest that multiple domains of hDaxx are required for repression, which implies that hDaxx may associate with multiple proteins in order to exert its repression activity. Therefore, we investigated this hypothesis by using
conventional chromatography to isolate endogenous hDaxx. We wanted to determine whether hDaxx associated with HDAC II, a protein that deacetylates acetylated histones, which results in transcriptional repression. In addition, we wanted to see if core histones and the protein Dek, a protein that associates with chromatin in vivo (Alexiadis et al., 2000), also associate with hDaxx. Daxx is a ubiquitously expressed protein that has transcriptional repression activity in a variety of cell lines (Hollenbach et al., 1999; Lehembre et al., 2001; Li et al., 2000a; Li et al., 2000b). Therefore, in order to facilitate the detection and immunoprecipitation of Dek using a FLAG affinity resin, we utilized U937T cells (Boer et al., 1998) that express endogenous hDaxx and stably express FLAG-epitope-tagged Dek (Dek-FLAG). Total cellular lysates from these cells were fractionated with a Sephacryl S-300 size exclusion column, and the presence of hDaxx, HDACII, acetylated histone H4, and Dek-FLAG was detected by western analysis. Examination of the individual fractions demonstrated that hDaxx eluted with an apparent molecular weight between 500 kDa and 700 kDa (Fig. 3A). This result indicated that hDaxx may associate with multiple proteins, because hDaxx eluted with a native molecular weight that is significantly larger than its denatured molecular weights of 70 kDa, 97 kDa, and 120 kDa (Hollenbach et al., 1999). In addition, HDACII, acetylated histone H4 and Dek-FLAG were also present in the fractions that contained hDaxx, indicating that these constituents co-fractionated with hDaxx from the Sephacryl S-300 column (Fig. 3A). The fractions that contained all four proteins (fractions 9-13, Fig. 3A) were then passed over a Resource Q anion exchange column and the proteins were eluted with a linear gradient from 0.15-1 M KCl. A western analysis demonstrated that the 70 kDa, nonphosphorylated form of hDaxx was eluted from the column 0.4-0.5 M KCl (Fig. 3B, closed circles), whereas the 120 kDa phosphorylated form was eluted at higher salt concentrations (Fig. 3B, open circles). Once again, HDACII and acetylated histone H4 were eluted with 0.4-0.5 M KCl, consistent with a co-fractionation of these proteins with the 70 kDa form of hDaxx (Fig. 3B). In addition, Dek-FLAG was found to elute to the same extent over a broad range of salt concentrations (Fig. 3B), which includes the fractions that contain hDaxx, HDAC II and acetylated histone H4. Therefore, because a fraction of Dek is present in the same fractions as hDaxx, HDAC II and acetylated histone H4, we believe that only a sub-population of Dek co-fractionates with these proteins.

The fractions that contained all four proteins (fractions 16-19) were pooled, concentrated and resolved on a Superdex HR-200 high-resolution gel-filtration column. As observed for the Sephacryl S-300 column, the majority of hDaxx eluted as a complex with an apparent molecular weight between 230-500 kDa with an average molecular weight of 360 kDa (Fig. 3C). In addition, both HDACII and Dek-FLAG, but not acetylated histone H4, eluted with hDaxx, confirming a co-fractionation of HDAC II and a sub-population of Dek-FLAG with hDaxx (Fig. 3C, lanes 12 and 13). To confirm that the observed molecular weight of 360 kDa is not due to the

---

**Fig. 2.** Daxx deletion mutant transcriptional repression activity. (A) The indicated hDaxx deletion mutants (10 fmol), with domains indicated as described for Fig. 1, or the GAL4 DBD alone were co-transfected into NIH3T3 cells with a chloramphenicol acetyl transferase (CAT) reporter construct containing one GAL4-DNA-binding site. Forty-eight hours after transfection, CAT activity was determined as described in the Materials and Methods. All values were normalized for co-transfected secreted alkaline phosphatase activity and are presented as the percentage of CAT activity in the absence of hDaxx. Errors represent the standard deviation from four independent determinations. (B) The protein expression levels for the GAL4-hDaxx deletion mutants. Equivalent amounts of total cellular lysate from NIH3T3 cells overexpressing each of the individual GAL4-hDaxx deletion mutants (30 μg) were separated by either 10% SDS-PAGE (left panel) or by a 4-15% SDS-PAGE gradient gel (right panel). The level of protein expression was determined by western analysis using an anti-GAL4-DBD monoclonal antibody.
Fig. 3. Co-fractionation of hDaxx, HDAC II, Dek and core histones. (A) Sephacryl S-300 size exclusion chromatography. Total cellular extracts of U937 cells (Boer et al., 1998) stably expressing FLAG-epitope tagged Dek were sonicated and separated by Sephacryl S-300 size exclusion chromatography. Each fraction was analyzed for the presence of hDaxx, Dek-FLAG, HDACII and acetylated histone H4 by western blot analysis. The elution volumes for molecular weight standards are noted below the gel. The amount of total protein (absorbance 280 nm, dotted line), hDaxx (circle, solid line), HDAC II (triangle, solid line) and acetylated histone H4 (square, solid line) in each fraction was determined by densitometry and is presented graphically as arbitrary densitometric units for each fraction. (B) Resource Q anion exchange chromatography. Fractions 9-13 of the Sephacryl S-300 column were fractionated over a Resource Q anion exchange column, and proteins were eluted with a 0.15-1 M KCl linear gradient. Equivalent volumes of protein from each fraction were DOC/TCA/acetone precipitated, separated on a 4-20% SDS-PAGE gel and analyzed for the presence of all four proteins. In addition to HDAC II and acetylated histone H4, both the 70 kDa form of hDaxx (closed circle, solid line) and the 120 kDa form of hDaxx (open circle, solid line) are presented. (C) Superdex HR 200 gel filtration chromatography. Fractions 16-19 of the Resource Q column were concentrated and fractionated over a Superdex HR 200 gel filtration column. Each fraction was analyzed for the presence of all four proteins as described above. In panels A-C the faster-migrating band observed for Dek-FLAG most probably consists of a degradation product as described by others (Alexiadis et al., 2000; McGarvey et al., 2000). (D) A Coomassie-blue-stained SDS-PAGE of the purified hDaxx complex. Lane 1 contains 1 µg of each of the purified histones H1, H2A, H2B, H3 and H4. Lane 2 contains 20 µg of DOC/TCA-precipitated protein from fraction 13 of the Superdex HR 200 column.
Dek-FLAG from the fractions of the Sephacryl S-300 column and HDAC II and core histones. First, we immunoprecipitated Dek-FLAG from the fractions of the Sephacryl S-300 column containing hDaxx (Fig. 3C, fraction 13) was deproteinized, and the presence of DNA was determined by 2% agarose gel electrophoresis. This analysis demonstrated that a small amount of DNA was present in this fraction and consisted of a fragment of approximately 180 bp, a size consistent with the DNA present in a mono-nucleosome (data not shown). In addition to the elution of hDaxx with an apparent molecular weight of 360 kDa, a small amount of hDaxx eluted with an apparent molecular weight of 670 kDa and, in addition to HDACII and Dek-FLAG, co-fractionated with acetylated histone H4 (Fig. 3C, lanes 10 and 11). The co-fractionation of acetylated histone H4 with only a small subset of hDaxx suggests that if hDaxx associates with acetylated histone H4, the association is of a transient nature, potentially because of the presence of HDAC II in these fractions. The co-fractionation of HDAC II with hDaxx and acetylated histone H4 would potentially bring HDAC II and acetylated histone H4 into close proximity, allowing the deacetylation of histone H4. Alternatively, co-fractionation may be fortuitous, with acetylated histone H4 and hDaxx being part of different large complexes.

The potential deacetylation of histones by HDAC II present in the complex suggests that non-acetylated histones may also co-fractionate with hDaxx, Dek and HDAC II in the 360 kDa fraction. Because commercially available antibodies did not recognize non-acetylated histones by western analysis in our hands, we determined the presence of non-acetylated histones in the 360 kDa hDaxx fractions by mass spectral analysis. The Coomassie-stained gel of proteins present in the peak hDaxx fraction from the Superdex HR-200 column demonstrated that in addition to several unidentified proteins of higher molecular weight, there was also the presence of bands consistent with the molecular weights of all histones (Fig. 3D). To confirm the identity of these proteins as the core histones H2A, H2B, H3 and H4, the bands corresponding to these proteins were excised from the gel, digested with trypsin, and the resulting peptide fragments were sequenced and identified by liquid chromatography and tandem mass spectral analysis. This analysis identified fragments corresponding to non-acetylated histones H2A, H2B, H3 and H4. A similar analysis of the band corresponding to histone H1 did not identify any peptide fragments corresponding to histone H1. Therefore, this result demonstrated that the core histones H2A, H2B, H3 and H4 co-fractionate with hDaxx, Dek and HDAC II in the 360 kDa hDaxx fraction.

Our results demonstrate that hDaxx, acetylated and non-acetylated core histones, HDAC II and a subpopulation of Dek-FLAG co-fractionate through a series of chromatographic separations. However, it is possible that their co-fractionation is merely fortuitous and that these proteins do not physically associate in vivo. Therefore we performed a series of co-immunoprecipitation experiments, which unlike co-fractionation depend on physical associations, using two independent components to confirm the association of hDaxx, Dek, HDAC II and core histones. First, we immunoprecipitated Dek-FLAG from the fractions of the Sephacryl S-300 column that contained all of these proteins using an anti-FLAG affinity column. After extensive washing of the precipitate and elution of the proteins from the FLAG antibody with a FLAG-specific peptide, a silver stain analysis of the bound proteins demonstrated that in addition to Dek-FLAG (Fig. 4A, right lane, top panel), the core histones H2A and H2B, and to a lesser extent H3 and H4, were efficiently co-immunoprecipitated by Dek (Fig. 4A, right lane, bottom panel). The co-immunoprecipitation of core histones was specific for Dek as they were only precipitated in the presence of Dek-FLAG (Fig. 4A), demonstrating an association between Dek and the core histones. The observed association of an abundance of H2A/H2B over H3/H4 with Dek-FLAG is identical to a previous report that demonstrated a stronger affinity of Dek for H2A/H2B (Alexiadis et al., 2000). A western analysis of the same proteins using either anti-hDaxx, anti-HDAC II or anti-acetylated histone H4 antibodies demonstrated that the immunoprecipitate also contained primarily the 70 kDa non-phosphorylated isoform of hDaxx, HDAC II and acetylated histone H4 (Fig. 4B, left lane). By contrast, the supernatant contained the 120 kDa phosphorylated isoform of hDaxx and HDAC II (Fig. 4B, right lane). Because the amount of protein loaded from the immunoprecipitate was five times more than the amount of protein loaded from the supernatant, we conclude that most of the 120 kDa isoform of hDaxx and a fraction of HDAC II were not retained on the column. The ability of Dek-FLAG to co-immunoprecipitate the 70 kDa form of hDaxx, Dek, HDAC II and core histones physically associate.

![Image](311x192 to 553x403)

A. Silver stain analysis of bound proteins. Bound proteins from lysates not expressing Dek-FLAG (left lane) and lysates expressing Dek-FLAG (right lane) were separated by 4-20% SDS-PAGE and visualized by silver staining. (B) Western blot analysis of bound proteins. Equal volumes of the bound (left lane) and unbound (right lane) fractions were separated by 4-20% SDS-PAGE, and the proteins were detected with a western blot analysis using anti-hDaxx, anti-HDAC II or anti-acetylated histone H4 antibodies.
of hDaxx, HDAC II and core histones therefore confirms that there is a direct association between Dek and these constituents.

Next we performed independent co-immunoprecipitation experiments using GAL4-hDaxx, as our hDaxx antibodies were raised against the SPT domain of hDaxx, the region demonstrated to interact with a variety of proteins (Chang et al., 1998; Hollenbach et al., 1999; Ishov et al., 1999; Kiriakidou et al., 1997; Li et al., 2000a; Pluta et al., 1998; Torii et al., 1999; Yang et al., 1999). 293T cells were transfected with full-length GAL4-hDaxx or GAL4 DBD alone and equivalent amounts of total cell lysate immunoprecipitated with the anti-GAL4 antibody. Consistent with the co-fractionation of hDaxx with both acetylated histone H4 and HDACII (Fig. 3), both proteins were co-immunoprecipitated with full-length GAL4-hDaxx (Fig. 5C,D, lane 2). This association was specific for hDaxx since neither acetylated histone H4 nor HDACII were detected when the identical co-immunoprecipitations were carried out with cells overexpressing GAL4 DBD alone (Fig. 5C and 5D, lane 1). This association was also independent of the presence of DNA since the same trace amount of low molecular weight DNA (~180 bp) was present regardless of whether the immunoprecipitation was performed with the anti-hDaxx or control antiserum (data not shown).

To determine which domains of hDaxx are required for the inclusion of chromatin-related proteins in the hDaxx complex, 293T cells were individually transfected with full-length GAL4-hDaxx or three N-terminal hDaxx deletion constructs (GAL4-hDaxxΔ1-132, GAL4-hDaxxΔ1-352, and GAL4-SPT) followed by immunoprecipitation of the total cell lysate. We observed an equivalent level of overexpression (Fig. 5A) and an equivalent level of immunoprecipitation (Fig. 5B) for all proteins. The GAL4-DBD immunoprecipitated protein signal was masked by the presence of the IgG light chain (Fig. 5B, lane 1); however, independent experiments using [35S]-Met metabolic labeling demonstrated an equivalent level of immunoprecipitation (data not shown). Despite the equivalent expression and immunoprecipitation of all hDaxx constructs, only full-length GAL4-hDaxx was able to co-precipitate acetylated histone H4 (Fig. 5C, lanes 2-5). This result demonstrated that the region surrounding the first paired amphipathic helix of hDaxx is required for the association of acetylated histone H4 with hDaxx. By contrast, it was found that all deletion constructs were able to co-immunoprecipitate HDAC II (Fig. 5D, lanes 2-5). Therefore, these results are consistent with the SPT domain being necessary for the association of HDAC II with hDaxx. Whether these interactions are mediated by a third protein is not known at present. However, despite this uncertainty, our results from the co-fractionation and two independent immunoprecipitation experiments demonstrate an association between the 70 kDa isoform of hDaxx, Dek-FLAG, HDAC II, acetylated histone H4 and the core histones H2A, H2B, H3 and H4.

**Daxx associates with active histone deacetylases**

Finally, we investigated the ability of hDaxx to immunoprecipitate active histone deacetylases. 293T cells were transfected with GAL4-hDaxx or GAL4 alone; equivalent amounts of total cell lysates were immunoprecipitated with the anti-GAL4 monoclonal antibody, and the immune complexes were tested for their ability to deacetylate [3H]acetetyl histone H4. Immunoprecipitates from cell lysates overexpressing GAL4-hDaxx contained two-fold higher levels of histone deacetylase activity compared with that of the positive control (Fig. 6, gray bars). This result is consistent with the association of hDaxx with histone deacetylase I (Li et al., 2000a) and II (Figs 3-5). This activity was specific for hDaxx as immunoprecipitates from cell lysates overexpressing GAL4 alone contained no deacetylase activity above background. The activity was also specific for histone deacetylases as the addition of the HDAC inhibitor sodium butyrate completely inhibited the release of [3H]acetate (Fig. 6, crosshatched bars).
Discussion

Originally identified as the cytoplasmic missing link between FasR and Jun N-terminal kinase (JNK) in Fas-dependent apoptosis (Chang et al., 1998; Yang et al., 1997), hDaxx has since been demonstrated to enhance apoptosis not in the cytoplasm but in the nucleus through its presence in PML oncopgenic domains (PODs) (Villunger et al., 2000; Zhong et al., 2000). Through its association in PODs it possibly functions as a transcriptional modulator that enhances Fas-induced apoptosis through effects on gene expression (Tori et al., 1999). The presence of hDaxx in PODs is mediated through an interaction with PML (Ishov et al., 1999; Li et al., 2000a). In addition to its presence in PODs, we and others have demonstrated that hDaxx exhibits transcriptional co-repression activity inhibiting the transcription factors Pax3 (Hollenbach et al., 1999) and ETS1 (Li et al., 2000b) through direct protein-protein interactions. The focus of the present study was to better understand the mechanism by which hDaxx exerts its repressive effect. Using a detailed structure-function analysis, chromatographic separation of endogenous hDaxx and its associated proteins and co-immunoprecipitation experiments, we have demonstrated that hDaxx physically associates with active HDAC II, which is essential for transcriptional repression, with the core histones H2A, H2B, H3 and H4 and with the chromatin associated protein Dek.

We present a minimal model to explain the transcriptional repression activity of hDaxx (Fig. 7) that is consistent with the data presented here and is based on previous models describing the repression activities of both Sin3 (Kadosh and Struhl, 1997; Nagy et al., 1997) and the retinoblastoma protein (Brehm and Kouzarides, 1999). In this model the post-translational modification status of the SPT-domain of hDaxx regulates its association with transcription factors such as Pax3 (Hollenbach et al., 1999) and ETS-1 (Li et al., 2000b), effectively bringing hDaxx to sites of active transcription. Through its presence at the site of active transcription, hDaxx would then be able to associate with acetylated histones present in the nucleosomes and Dek that is associated with chromatin (Kappes et al., 2001). Through its association with the SPT-domain of hDaxx, histone deacetylases would also be brought to the site of active transcription. As a consequence, nucleosomes in the vicinity of the site of active transcription will have the histone tails deacetylated, allowing the deacetylated tail to bind to DNA, thereby leading to an inactive chromatin structure and transcriptional repression.

Our proposed working model of transcriptional repression by hDaxx is based on the following evidence. First, we have previously demonstrated that hDaxx exists in at least three distinct isoforms, with apparent molecular weights of 70 kDa, 97 kDa and 120 kDa (Hollenbach et al., 1999). Of these three isoforms, we have demonstrated that the 120 kDa non-phosphorylated isoform of hDaxx is phosphorylated (Hollenbach et al., 1999). The phosphorylation status of hDaxx is responsible for regulating its association with different proteins. For example, the transcription factor Pax3 specifically interacts with the non-phosphorylated 70 kDa isoform of hDaxx, resulting in the repression of Pax3 transcriptional activity (Hollenbach et al., 1999). Conversely, PML, a protein present in PODs, interacts specifically with the phosphorylated 120 kDa isoform of hDaxx, resulting in an enhancement of apoptosis potentially by functioning as a transcriptional modulator that enhances Fas-induced apoptosis through effects on gene expression (Ishov et al., 1999; Li et al., 2000a; Torii et al., 1999; Zhong et al., 2000). In both of these cases the SPT domain of hDaxx, one of the primary regions of hDaxx phosphorylation (A.D.H. and G.G., unpublished), mediates the interaction between hDaxx and Pax3 (Hollenbach et al., 1999) and PML (Ishov et al., 1999). In addition, we demonstrate that only the 70 kDa non-phosphorylated isoform of hDaxx associates with HDAC II, Dek and core histones (Figs 3 and 4). Therefore, taken together, these results support the hypothesis that the phosphorylation status of hDaxx regulates its association with chromatin, thereby regulating the repression activity of hDaxx.

Second, we have demonstrated that endogenous hDaxx elutes from size exclusion chromatography columns with an apparent molecular weight of approximately 360 kDa (Fig. 3A,C), suggesting that it associates with multiple proteins. Through a series of chromatographic separations we have demonstrated that the 70 kDa isoform of hDaxx co-fractionates with components of chromatin such as core histones, proteins that associate with chromatin such as Dek and proteins that are critical for transcriptional repression such as HDAC II (Fig. 3). We have also demonstrated that hDaxx physically associates with HDAC II, Dek and acetylated core histones, as seen by the co-immunoprecipitation of these components by either hDaxx (Fig. 5) or Dek-FLAG (Fig. 4). It is possible that hDaxx
interacts indirectly with HDAC II and core histones through non-specific association with DNA. However the ability to co-immunoprecipitate acetylated histone H4 and HDAC II independently of the presence of DNA supports the conclusion that the association occurs through protein-protein interactions. Finally, we have demonstrated that hDaxx is capable of immunoprecipitating histone deacetylase activity (Fig. 6). The association of hDaxx with histone deacetylase activity is consistent with the co-fractionation of HDAC II with hDaxx (Fig. 3) and the ability of hDaxx to co-immunoprecipitate HDAC I (Li et al., 2000a) and HDAC II (Figs 4 and 5). Therefore, by bringing histone deacetylase activity to sites of active transcription, hDaxx facilitates the deacetylation of histone tails in nucleosomes, allowing the otherwise extended and acetylated histone tails to bind to DNA. Through the binding of the histone tails to DNA, they would then prevent the access of elements required for transcription, which would result in repression of transcriptional activity.

At present the exact function of Dek in hDaxx-mediated repression is not clear. Our data demonstrating the co-immunoprecipitation of hDaxx by Dek (Fig. 4) would suggest that a direct association exists between these two proteins. However we cannot exclude the possibility that this association is secondary in nature, being mediated through a mutual association with the core histones present in chromatin. Regardless of whether the association of Dek with hDaxx is direct or secondary, our results suggest that in addition to its reported effects on DNA replication (Alexiadis et al., 2000) and its presence in the exon-exon junction complex (Le Hir et al., 2000; Le Hir et al., 2001), Dek may also have a function in transcriptional repression. Dek has been reported to associate with chromatin in vivo (Kappes et al., 2001) and to alter the superhelical density of DNA in chromatin in vitro, inhibiting the access of proteins and replication factors to the DNA template (Alexiadis et al., 2000). Therefore it is conceivable that in a similar manner Dek may also function during transcription by altering the structure of chromatin once histone tails have been deacetylated and preventing the access of transcription factors to the DNA template.

It is unlikely that hDaxx-mediated deacetylation and subsequent repression of transcription is a continuous process in vivo. Rather, it is likely that the deacetylation and transcriptional repression are regulated via additional signals. Although we have no data to indicate the identity of these potential signals, one possibility is that additional post-translational modifications that regulate the interactions of hDaxx with specific transcription factors, such as Pax3 or Ets1, could trigger the deacetylase activity. Alternatively, its association with transcription factors within a particular promoter context may regulate the activity of hDaxx. For example, although no reports have been published that describe hDaxx-mediated transcriptional activation, the association of acetylated histones with the hDaxx complex could indicate a role for hDaxx in transcriptional activation in certain promoter settings. However, regardless of whether the transcriptional regulatory activity of hDaxx is modulated by interactions with transcription factors or by its presence within a specific promoter context, our results demonstrate that transcriptional repression by hDaxx is mediated through its association with core histones, HDAC II and Dek. Our present efforts are aimed at identifying potential signals that regulate hDaxx transcriptional repression activity.
All mass spectral analyses were performed in the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital. We thank Erik Bonten for technical assistance with the isolation of endogenous hDaxx, and Joe Vaccaro for his critical reading of the manuscript. This work was supported, in part, by NIH grants CA71907-05 and CA76480-03, the Cancer Center (CORE) support grant CA-21765 and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children’s Research Hospital.

References


