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Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs)

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Daxx was first identified as a protein that binds the cytosolic domain of Fas and links this receptor to an apoptosis pathway involving activation of Jun N-terminal kinase (JNK). We show here that cells overexpressing the human homolog of Daxx (hDaxx) display enhanced sensitivity to apoptosis induced by Fas but not by several other cell death stimuli. hDaxx-mediated enhancement of Fas-induced apoptosis was correlated with accelerated activation of caspases but not with JNK induction. Although specifically enhancing Fas function, hDaxx does not bind Fas and instead is found in the nucleus where it localizes to PML oncogenic domains (PODs). Moreover, the hDaxx protein also exhibits the ability to repress transcription. Mutagenesis studies demonstrated a correlation between the localization of hDaxx to PODs and its ability to enhance Fas-induced cell death. Arsenic trioxide (As_2O_3), an agent that accentuates POD formation, collaborated synergistically with overexpression of hDaxx to increase cellular sensitivity to Fas-induced apoptosis. Taken together, these findings argue that hDaxx promotes sensitivity to Fas from a nuclear location, probably by modulating the transcription of genes involved in Fas-induced caspase activation and apoptosis.

Keywords: caspase activation/Fas-induced apoptosis/hDaxx/PML oncogenic domains

Introduction

The Daxx protein was first identified in yeast two-hybrid screens for cDNAs encoding proteins capable of binding the cytosolic domain of Fas, an apoptosis-inducing member of the tumor necrosis factor (TNF) receptor family (Yang *et al*., 1997). Mouse Daxx (mDaxx) reportedly binds the death domain of Fas and also associates with Ask-1, a MAP3K that triggers the Jun N-terminal kinase (JNK) pathway (Chang *et al*., 1998). Overexpression of mDaxx induces JNK activation, whereas expression of mDaxx fragments containing the C-terminal domain implicated in Fas binding inhibits both Fas-induced JNK activation and Fas-induced apoptosis (Yang *et al*., 1997; Chang *et al*., 1998). These and other findings have thus suggested a role for mDaxx in a Fas-mediated signal transduction pathway that operates independently of and in parallel with the Fas-binding protein Fadd and its chief molecular target caspase-8 (Chang *et al*., 1999).

The apparent primate and human homologs of Daxx have been identified during cDNA library screens for proteins capable of binding DNA promoters in yeast one-hybrid assays and for proteins that bind the centromere-associated protein CENP-C or that bind DNA methyltransferase-I in yeast two-hybrid assays (Kiriakidou *et al*., 1997; Pluta *et al*., 1998; Michaelson *et al*., 1999). The human Daxx protein (hDaxx) is 72% identical to mDaxx in its overall predicted amino acid sequence. The mouse, primate and human proteins contain at least two candidate nuclear localization sequences (NLS) and an acidic domain similar to some transcriptional regulators (Kiriakidou *et al*., 1997; Yang *et al*., 1997; Pluta *et al*., 1998). The Daxx protein reportedly resides primarily in the nucleus (Pluta *et al*., 1998). The relevance of hDaxx to Fas-induced apoptosis has not been addressed previously.

Sensitivity to Fas-induced apoptosis was reported recently to be regulated by PML, a nuclear protein that localizes to nuclear substructures known as PML oncogenic domains (PODs). Mice with homozygous disruptions of their *pml* genes display resistance to apoptosis induced by Fas, as well as by X-irradiation and ceramide (Wang *et al*., 1998b). Overexpression of the PML protein also reportedly induces cell death (Quignon *et al*., 1998). The *pml* gene functions as a tumor suppressor in mice (Wang *et al*., 1998a). In human acute promyelomonocytic leukemias (APLs), the *pml* gene is commonly fused to the *retinoic acid receptor*-α (*RAR*α) gene as a result of t(15;17) chromosomal translocations, generating PML–RARα chimeric proteins (de The *et al*., 1991; Kakizuka *et al*., 1991). The PML–RARα protein forms hetero-oligomers with endogenous PML and disrupts the formation of PODs (Dyck *et al*., 1994), thus linking loss of PML localization to PODs with oncogenesis. APL responds clinically to all-*trans* retinoic acid (ATRA), resulting in APL cell differentiation and apoptosis, which correlates with relocalization of PML to PODs and degradation of the PML–RARα oncoprotein (Soignet *et al*., 1998). Arsenicals have also proven effective in the treatment of APL, including ATRA-resistant leukemias. Arsenic trioxide (As_2O_3) enhances POD formation in the nuclei of APL cells in association with induction of leukemia cell differentiation and apoptosis (Chen *et al*., 1996a). Arsenicals also increase the sizes of PODs in cells lacking PML–RARα fusion proteins, and induce the redistribution of PML to PODs in association with covalent modification of PML with the ubiquitin-like protein SUMO-1 (Sternsdorf *et al*., 1997; Zhu *et al*., 1997; Muller *et al*., 1998). Interestingly, overexpression of SUMO-1 has also been reported to suppress apoptosis induced by Fas (Okura *et al*., 1996). Although the functions of the PML protein both in its normal context and when fused with RARα

Table I. Yeast two-hybrid analysis

Lex	B42	−Leu	β-gal
Caspase-10	DaxxC	+	+
Caspase-8	DaxxC	+	+
Fas-C tail	DaxxC	−	+
Fas	DaxxE	−	−
Fas	DaxxEC	−	+
Fas	Fadd	+	+
Fas	caspase-10	−	−
DaxxEC	vector	+	+
DaxxEC	Bax	+	+
DaxxC	Fax-C tail	−	−
DaxxC	caspase-10	−	−

Plasmids producing either LexA DNA-binding domain fusion proteins (left) or B42 trans-activation fusion proteins (right) were cotransformed into EGY48 strain containing *LEU2* and *lacZ* reporter genes. DaxxC, DaxxE and DaxxEC contain residues 622–740, 493–625 and 493–740 of the hDaxx protein, respectively (see Materials and methods for details on other plasmids). Transformed cells which grew on leucine-deficient media within 3 days were scored as positive (+). The β-galactosidase activity of each clone was tested by filter assay and scored as positive (blue) versus negative (white) after 90 min.

remain unclear, evidence supporting a role for PML in transcriptional activation or repression has been obtained (Vallian *et al*., 1997; La Morte *et al*., 1998; Doucas *et al*., 1999). Altogether, the present information about PML strongly suggests an important role for this nuclear protein in tumor suppression by promoting programmed cell death through effects on gene transcription.

In this report, we explored the relevance of hDaxx to apoptosis induced by Fas and other stimuli. Although hDaxx specifically enhanced Fas-mediated cell death, we could find no evidence that this protein associates with Fas in cells. Instead, hDaxx co-localizes with PML within nuclear PODs. Moreover, Fas stimulation fails to induce relocalization of Daxx from PODs. The C-terminal domain corresponding to the putative Fas-binding region of this protein (Yang *et al*., 1997) was found to be required for: (i) enhancement of Fas-induced apoptosis; (ii) localization of hDaxx to PODs; and (iii) optimal repression of transcription by Daxx. Taken together, these observations indicate that hDaxx regulates cellular sensitivity to Fas from PODs within the nucleus, possibly by functioning as a transcriptional modulator that enhances Fas-induced apoptosis through effects on gene expression.

Results

The hDaxx protein does not bind Fas

During two-hybrid screens for caspase-10 binding proteins, we obtained two cDNAs encoding the C-terminal region of hDaxx representing residues 493–740 and 622–740. However, additional analysis of these fragments of hDaxx (Table I) as well as full-length hDaxx (not shown) in two-hybrid experiments demonstrated that hDaxx displays non-specific interactions with several proteins, including caspase-10, caspase-8, Bax and Fadd. Interestingly, however, hDaxx failed to interact with the cytosolic domain of hFas in two-hybrid assays, contrary to expectations (Yang *et al*., 1997). The failure of hDaxx to associate with the cytosolic domain of hFas was evident in both orientations in two-hybrid assays (Table I) and was not attributable to a failure to produce either protein in yeast, as determined by immunoblotting (not shown). In contrast, hFas did display interactions with the adaptor protein Fadd, but not with caspase-8 or -10, consistent with previous reports (reviewed in Salvesen and Dixit, 1997; Wallach *et al*., 1997).

To evaluate further the potential interaction of hDaxx with Fas, co-immunoprecipitation experiments were performed. For these experiments, a cDNA encoding the full-length human Daxx protein was generated by RT–PCR from Jurkat T-cell mRNA and cloned into mammalian expression plasmids with either HA- or Flag-epitope tags (pcDNA3-HA, pCI-FLAG). 293T cells were then transiently co-transfected with plasmids encoding HA-tagged hFas cytosolic domain (Cyt) and either Flag-tagged hDaxx or Flag-tagged Fadd. As shown in Figure 1A, HA–Fas (Cyt) was contained in Flag–Fadd immune complexes, but not in Flag–Daxx. Immunoblot analysis of lysates prepared from these same transfected cells confirmed the production of similar amounts of Flag–Fadd and Flag–hDaxx, excluding differences in the levels of these proteins as an explanation for their differential association with the cytosolic domain of Fas.

Because experiments using the cytosolic domain of Fas revealed no association with hDaxx, we considered the possibility that hDaxx might only bind to Fas within the context of the full-length receptor. However, when expression plasmids encoding full-length hFas were co-transfected into 293T cells with HA–hDaxx or HA–Fadd, again Fadd was readily detected in anti-Fas immune complexes while hDaxx was not (Figure 1B). Immunoblot analysis of the lysates from these transfected cells confirmed the production of comparable amounts of HA–hDaxx and HA–Fadd, thus differences in the amounts of Fadd and Daxx produced in cells cannot account for the failure to detect association of hDaxx with Fas. Similar results were obtained regardless of whether Fas-L was added to the cultures prior to lysis (not shown), indicating that hDaxx association with Fas is also not ligand inducible. Overexpression of hDaxx also did not enhance or interfere with ligand-induced association of Fadd with Fas, as determined by co-immunoprecipitation experiments (unpublished observations).

Although unable to bind Fas, hDaxx was capable of self-associating, based on co-immunoprecipitation experiments in which HA–hDaxx and Flag–hDaxx were co-expressed in 293T cells. As shown in Figure 1C, immunoprecipitation with anti-Flag followed by SDS–PAGE and immunoblot analysis with anti-HA antibody provided evidence of self-association of hDaxx. These data therefore provide an additional control suggesting that the hDaxx protein expressed in these experiments is competent to bind some proteins (i.e. itself), though displaying no apparent affinity for Fas. The region within hDaxx required for self-association was mapped to the N-terminal portion of the protein (residues 1–251) using a panel of hDaxx mutants with progressive deletions from the C-terminus, including full-length Daxx(1–740), Daxx(1–625), Daxx(1–502) and Daxx(1–251). In contrast, the C domain of Daxx previously implicated in Fas binding (residues 493–740) did not associate with full-length Daxx in co-immunoprecipitation experiments. The lack of interaction of the HA–Daxx(493–740) fragment with full-length Flag–Daxx also provides

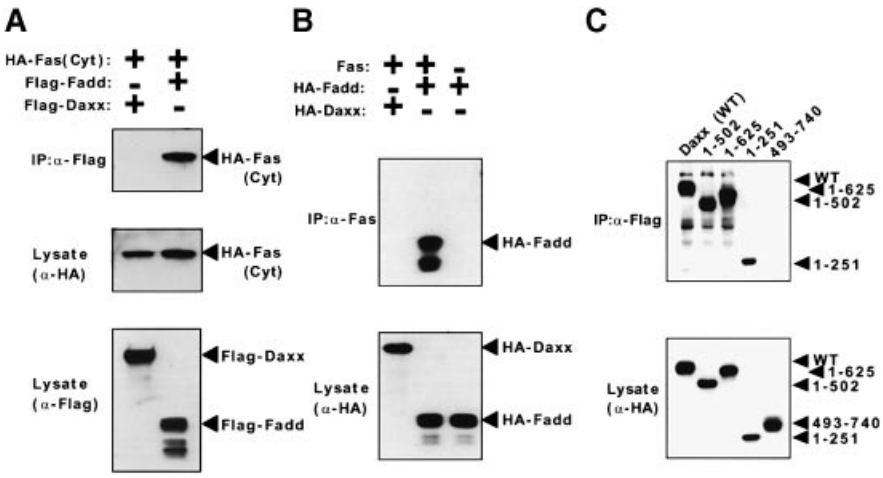

Fig. 1. hDaxx does not interact with human Fas. (**A**) 293T cells were transiently transfected with the indicated combinations of expression vectors encoding Flag-tagged Daxx or Flag–Fadd and HA-tagged cytoplasmic domain (Cyt) of human Fas. After 24 h, lysates were prepared and immunoprecipitated (IP) with anti-Flag monoclonal antibody M2-conjugated agarose. The immunoprecipitates as well as an aliquot of the original lysates were analyzed by immunoblotting using anti-HA rat monoclonal antibody (3F10) with ECL-based detection. (**B**) 293T cells were co-transfected with expression plasmids encoding full-length Fas, HA–hDaxx or HA–Fadd. After 20 h, lysates were prepared and immunoprecipitated (IP) with anti-Fas monoclonal antibody (DX2). Both lysates and immunoprecipitants were analyzed by immunoblotting with anti-HA antibody. (**C**) 293T cells were transiently transfected with expression plasmids encoding full-length wild-type (WT) Flag–hDaxx or HA–hDaxx (WT; lane 1), Daxx1–502 (lane 2), Daxx1–625 (lane 3), Daxx1–251 (lane 4) or Daxx493–740 (lane 5). After 24 h, cell lysates were prepared and immunoprecipitated (IP) with anti-Flag monoclonal antibody-conjugated agarose. Both immunoprecipitants (top) and lysates (bottom) were analyzed by immunoblotting with anti-HA antibody.

an internal negative control, suggesting that the observed interaction of Flag–Daxx and HA–Daxx is specific.

hDaxx is a nuclear protein whose location is unaltered by Fas

Confocal immunofluoresence microscopy was used to analyze the intracellular location of the hDaxx protein. For these experiments, Flag-epitope-tagged hDaxx was expressed in HT1080 fibrosarcoma, HEK293 kidney epithelial cells or GM701 fibroblasts and these cells were then fixed and stained with anti-Flag antibodies. In all cell lines examined, Flag–Daxx immunofluorescence was present exclusively in a nuclear location, with a speckled pattern (Figure 2A). These observations are consistent with the presence within both the human and mouse Daxx proteins of two predicted NLS, and corroborate observations by others (Kiriakidou *et al*., 1997; Pluta *et al*., 1998). For comparison with hDaxx, immunofluorescence microscopic analysis was also performed using cells transfected with plasmids encoding proteins that are known to be cytosolic, namely Flag–Bcl-2 or Flag–Raidd. In contrast to cells expressing Flag–Daxx, anti-Flag staining of cells expressing Flag–Bcl-2 protein revealed predominantly cytosolic immunofluorescence in a pattern consistent with the known association of Bcl-2 with intracellular membranes (Figure 2B). In cells expressing Flag–Raidd, an adaptor protein implicated in TNF signaling (Ahmad *et al*., 1997; Duan and Dixit, 1997), anti-Flag immunostaining again revealed a cytosolic location, with accentuated immunofluorescence in the vicinity of the plasma membrane (Figure 2B).

Using confocal immunofluorescence microscopy, we next examined the effects of stimulating cells through Fas on the intracellular distribution of Daxx, inquiring whether activation of Fas might induce translocation of hDaxx to the plasma membrane region. Comparisons were made with immunostaining for cytochrome *c*, which is known to undergo translocation from mitochondria into the cytosol during apoptosis (reviewed in Reed, 1997; Green and Reed, 1998). For these experiments, we employed HT1080 cells that had been stably transfected with a plasmid producing HA-tagged Daxx and used the mouse IgM anti-Fas antibody CH11 to cross-link Fas. As shown in Figure 2C, when hDaxx-overexpressing cells were cultured with anti-Fas antibody for 12 h, the hDaxx staining pattern was unaltered as compared with untreated control cells. In contrast, cytochrome *c* immunostaining changed from a punctate pattern typical of mitochondrial association to a diffuse cytosolic distribution (Figure 2C). When taken together with the co-immunoprecipitation experiments, these observations strongly suggest that hDaxx is not recruited to Fas receptors and remains within the nuclei of cells undergoing Fas-induced apoptosis.

hDaxx specifically enhances Fas-induced apoptosis

Although hDaxx failed to interact with Fas, we observed that overexpression of this protein did enhance Fas-induced apoptosis reproducibly, consistent with prior observations (Yang *et al*., 1997). Figure 3, for example, presents results using HT1080 cells that had been stably transfected with plasmids producing either HA- or Flag-tagged hDaxx. Controls consisted either of untransfected HT1080 cells or HT1080 cells that had been stably transfected with a control pcDNA3 plasmid (Neo). Each of these stably transfected lines represents a pool of multiple stable transfectants, thus avoiding clonal bias. Immunoblotting confirmed the production of the Flag–hDaxx and HA–Daxx proteins. As shown, overexpression of either HA–hDaxx or Flag–hDaxx increased the percentage of cells killed by anti-Fas antibody (CH11) over a wide range of concentrations (10 ng/ml–1 μg/ml), as determined by Trypan Blue dye-exclusion assays.

Similar observations were made in transient transfection

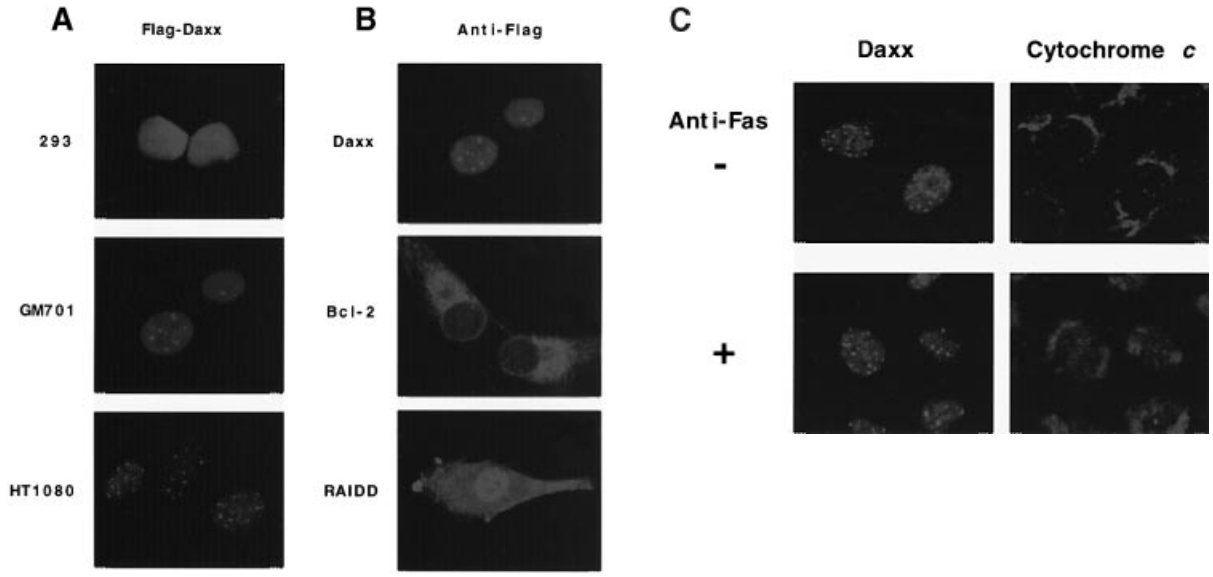

Fig. 2. hDaxx is located in the nucleus. (**A**) 293, GM701 or HT1080 cells were transfected with expression plasmids encoding Flag-tagged hDaxx. After 48 h, cells were fixed and stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. (**B**) Plasmids encoding Flag-tagged Daxx, Bcl-2 or RAIDD were transfected into GM701 cells. At 2 days after transfection, cells were fixed and stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. (**C**) Fas stimulation does not alter the intracellular localization of Daxx. HT1080/Daxx stable cell lines were incubated with CH11 anti-Fas monoclonal antibody (IgM) for 12 h. After fixation, permeabilized cells were incubated with anti-HA rat monoclonal antibody (3F10) and anti-cytochrome *c* mouse monoclonal antibody (6H2.B4) followed by FITC-labeled anti-rat IgG and Texas Red-labeled anti-mouse IgG. The stained cells were analyzed by confocal microscopy.

experiments in which apoptosis was induced by overexpression of the Fas proteins. Figure 4A, for example, presents results from transient transfection assays using 293 cells where plasmids encoding Fas or other apoptotic proteins were co-transfected with either a control vector or pcDNA3-HA-Daxx. For assessing apoptosis, cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI), using co-transfection of a green fluorescence protein (GFP)-producing plasmid to identify successfully transfected cells. As shown, hDaxx enhanced Fas-induced apoptosis, although overexpression of hDaxx by itself did not induce apoptosis (Figure 4A). Similar results were obtained using human breast cancer MCF7/Fas cells, human prostate cancer ALVA31 cells and human fibrosarcoma HT1080 cells (data not shown). The hDaxx-mediated enhancement of Fas-induced apoptosis was highly reproducible over a total of 10 independent transfection experiments, ranging from an ~15 to 50% increase in apoptosis (mean 34 ± 11%; $p = 0.01$) compared with cells transfected with Fas plasmid alone.

Enhancement of apoptosis by overexpression of hDaxx appeared to be selective for Fas. As shown in Figure 4A, hDaxx did not modulate apoptosis induction by overexpression of Fadd, pro-caspase-8 or pro-caspase-10, suggesting that hDaxx operates at a point proximal to Fadd in the Fas-induced apoptosis cascade. Moreover, while hDaxx enhanced apoptosis induced by overexpression of Fas, it did not significantly alter apoptosis induction by overexpression of other TNF-family death receptors, including TNFR1 (Figure 4A), DR4 or DR5 (not shown).

Similar conclusions about the selectivity of hDaxx effects on Fas-induced apoptosis were reached using the HT1080 stable transfectants described above. Apoptosis induced in these cells by anti-Fas antibody was augmented by overexpression of HA–Daxx, whereas cell death induced by TNF-α in combination with cycloheximide

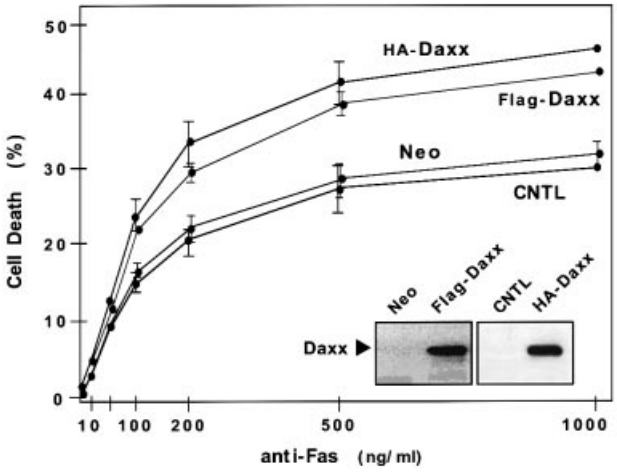

Fig. 3. hDaxx enhances Fas-induced apoptosis in HT1080 cells. Untransfected HT1080 cells (CNTL) and HT1080 cells that had been stably transfected with pcDNA3 (Neo), Flag–Daxx or HA–Daxx were incubated with various concentrations of anti-Fas antibody. The percentage of dead cells was determined by Trypan Blue dye uptake. The insets show immunoblot analysis of cells lysates normalized for total protein content (50 μg/lane) using anti-HA or anti-Flag antibodies.

(CHX) was unaffected (Figure 4B). Also shown are experiments in which apoptosis was induced by the combination of anti-Fas antibody and CHX, which allows a lower concentration of anti-Fas antibody to be employed. Again, a higher percentage of the cells overexpressing HA–Daxx underwent apoptosis after treatment with anti-Fas plus CHX compared with control untransfected HT1080 cells (Figure 4B). The ability of Daxx to enhance Fas-induced apoptosis even in the presence of CHX suggests that gene expression is not required.

Overexpression of Daxx also did not alter the sensitivity

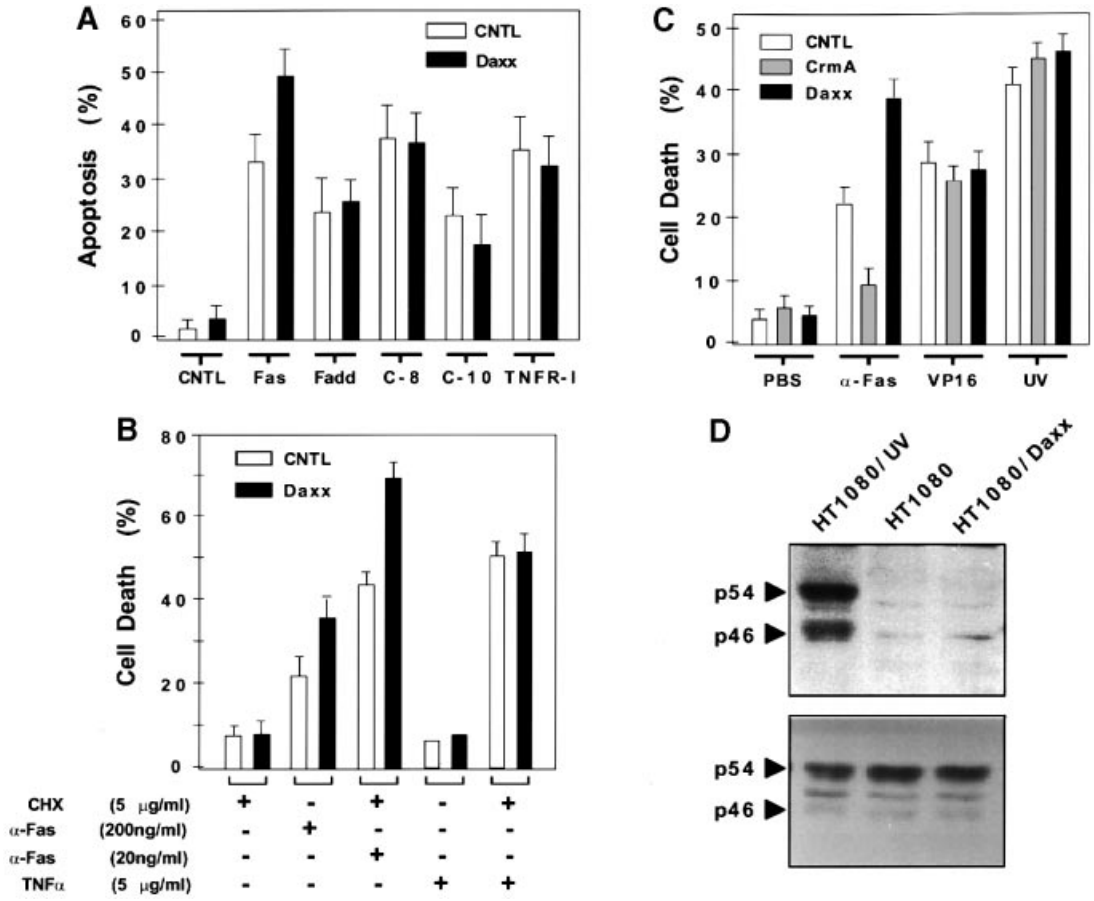

Fig. 4. hDaxx specifically enhances Fas-induced apoptosis but does not elevate JNK activity. (**A**) 293 cells were transiently transfected with 0.5 μg of pcDNA3 control plasmid (white bars) or Daxx expression plasmid (black bars), together with 0.1 μg pEGFP and 0.3 μg plasmids encoding Fas, TNFR1, Fadd, caspase-8 or caspase-10, or 0.3 μg 'empty' control plasmid. One day later, cells were recovered and the percentage of apoptotic cells was determined by DAPI staining (mean ± SE; $n = 3$) among GFP-positive cells. (**B**) HT1080 control (white bars) or hDaxx (black bars) stable cell lines (1×10^6) were incubated with anti-Fas monoclonal antibody (CH11), 5 μg/ml of TNF-α, with or without 5 μg/ml cycloheximide (CHX). After 24 h, both floating and adherent cells were recovered and subjected to Trypan Blue dye exclusion assay (mean ± SE; $n = 3$). (**C**) HT1080 stable cell lines expressing CrmA or HA–Daxx were produced. Cells (1×10^6) were incubated with anti-Fas monoclonal antibody CH11 (0.3 μg/ml) or 20 μg/ml VP16 (etoposide) for 24 h or treated with 40 J/m^2 UV radiation. After 24 h, both floating and adherent cells were recovered and subjected to Trypan Blue dye exclusion assay (mean ± SE; $n = 3$). (**D**) Lysates were generated from untreated HT1080 control or hDaxx-overexpressing cells or from HT1080 cells that had been exposed to 40 J/m^2 UV radiation. Lysates (80 μg total protein) were subjected to SDS–PAGE and immunoblotting and phosphorylated JNK was detected using an anti-phospho-SAPK/JNK antibody that recognizes phospho Thr183 and Tyr185 (upper panel). Total JNK protein levels were determined by immunoblotting with anti-SAPK/JNK antibody (lower panel). Arrowheads indicate the p54 and p46 isoforms of JNKs.

of HT1080 cells to cell death induction by apoptotic stimuli that operate through death receptor-independent mechanisms such as UV irradiation or the anticancer drug VP16 (etoposide) (Figure 4C). As a control, results are also shown for transfected HT1080 cells which express the cowpox CrmA protein, a potent inhibitor of caspase-8 (Zhou *et al*., 1997). While CrmA suppressed apoptosis induced by anti-Fas antibody, it did not significantly affect apoptosis induced by UV or VP16 (Figure 4C). Analogously, while hDaxx increased apoptosis induction by anti-Fas, it did not substantially alter apoptotic responses to UV or VP16.

Because previous investigations of Daxx have reported that overexpression of mDaxx induces JNK activation (Yang *et al*., 1997; Chang *et al*., 1998), we also compared the levels of JNK activity in HT1080 cells overexpressing hDaxx with cells that had been treated with UV irradiation, a known stimulator of stress-kinase pathways leading to JNK activation (Hibi *et al*., 1993). Phosphorylation of JNK on threonine 183 and tyrosine 185 correlates with activation of this kinase, and was used as a surrogate marker of JNK activation for most experiments, although similar results were obtained by the solid-phase *in vitro* kinase assays using immobilized glutathione *S*-tranferase (GST)–c-Jun fusion protein as a substrate (data not shown). As shown in Figure 4D, levels of phosphorylated JNK were not elevated in HT1080 cells stably overexpressing hDaxx compared with control cells, as determined by immunoblotting using a phosphospecific anti-JNK antibody that recognizes the p46 and p54 isoforms of JNK. Stimulation with the anti-Fas antibody CH11 also did not result in detectable increases in JNK activity in these particular cells (not shown). In contrast, UV-irradiated HT1080 cells exhibited a striking increase in JNK phosphorylation. Immunoblot analysis of the same lysates using a phosphorylation-independent anti-JNK antibody confirmed similar total levels of the JNK proteins in these cells (Figure 4D). Similar conclusions were reached using other cell lines in which hDaxx was transiently overexpressed, also suggesting that overexpression of hDaxx

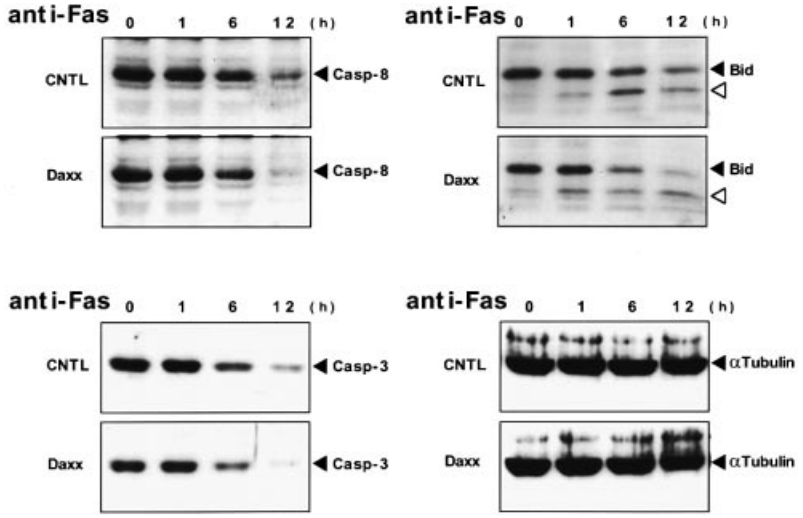

Fig. 5. hDaxx accelerates Fas-induced processing of caspases. HT1080 control or Daxx-overexpressed cells were incubated with anti-Fas antibody (0.5 μg/ml) for 0, 1, 6 or 12 h. Cell extracts were prepared and subjected to SDS–PAGE followed by immunoblotting and incubation with antibodies that recognize pro-caspase-8, pro-caspase-3, Bid or α-tubulin. Arrowheads in the Bid panel indicate full-length and caspase-cleaved forms of Bid.

does not induce transient increases in JNK activity (not shown). We cannot, however, exclude the possibility that hDaxx might promote increased activation of JNK under some conditions not explored here.

hDaxx accelerates Fas-induced activation of caspases

It has been proposed that Fas can trigger two parallel apoptotic pathways: one involving stress-kinase activation via Daxx (Yang *et al*., 1997) and another in which the adaptor protein Fadd recruits pro-caspase-8 to Fas death domains and initiates a cascade of proteolysis (Salvesen and Dixit, 1997; Wallach *et al*., 1997). Since we found no evidence that hDaxx regulates JNK activity, we turned our attention to the caspase-mediated pathway for apoptosis that Fas is known to stimulate. Using stably transfected HT1080 cells (Neo versus hDaxx), we compared the kinetics of Fas antibody-induced processing of pro-caspase-8, as well as proteolytic processing of the downstream caspase-8 substrates, Bid (Li *et al*., 1998; Luo *et al*., 1998) and pro-caspase-3 (Stennicke *et al*., 1998).

As shown in Figure 5, pro-caspase-8 was depleted from Fas-stimulated HT1080-hDaxx cells slightly faster than in HT1080 control cells. At 12 h after anti-Fas antibody stimulation, for example, nearly all the pro-caspase-8 has been consumed in hDaxx-overexpressing cells, whereas residual pro-caspase-8 remained in the control cells. Unfortunately, detection of the proteolytically processed fragments of caspase-8 was not possible using available antibodies. However, the observation that loss of pro-caspase-8 from Fas-stimulated cells was completely prevented by culturing the cells in the presence of 100 μM zVAD-fmk, a broad-spectrum inhibitor of caspases (Armstrong *et al*., 1996), indicated that proteolysis rather than changes in gene expression or other events was responsible (not shown). Analysis of the same blot with anti-tubulin antibody confirmed loading of equivalent amounts of total protein for all samples (Figure 5).

Although differences in the rates of Fas-induced pro-caspase-8 processing in hDaxx-overexpressing versus control cells were somewhat subtle, the kinetics of processing of caspase-8 substrates (Bid and pro-caspase-3) were clearly accelerated in hDaxx-overexpressing compared with control HT1080 cells. For example, the appearance of the cleaved form of Bid was clearly evident in hDaxx-overexpressing cells within 1 h after Fas stimulation and most of the endogenous Bid protein had been cleaved by 12 h in these cells, whereas Bid cleavage was barely detectable at 1 h after Fas stimulation in control cells and much of the protein remained unprocessed at 12 h (Figure 5). Processing of pro-caspase-3, another documented direct substrate of caspase-8 (Stennicke *et al*., 1998), was also accelerated in cells overexpressing hDaxx compared with control HT1080 cells (Figure 5). Moreover, the accumulation of caspase-3-like protease activity was also accelerated in hDaxx-overexpressing HT1080 cells compared with control untransfected cells, based on cleavage of the fluorigenic substrate Asp-Glu-Val-Asp-aminofluorocoumarin (DEVD-AFC) (not shown). Similar results were obtained using MCF7/Fas cells (unpublished observations). Although not presented here, the rate of caspase-3 processing and activation was not dissimilar in hDaxx-overexpressing and control cells when apoptosis was induced using a Fas-independent stimulus, staurosporine, thus demonstrating the specificity of these results. Taken together, these findings suggest that hDaxx overexpression enhances Fas signaling through its effects on caspases.

hDaxx and PML co-localize

The nuclear structures (speckles) in which hDaxx was localized by confocal immunofluorescence microscopy are reminiscent of PODs. The PML tumor suppressor protein localizes to PODs, and has been implicated in the control of sensitivity to apoptosis induction by Fas and some other stimuli (Quignon *et al*., 1998; Wang *et al*., 1998b). Using HT1080 cells that had been co-transfected with plasmids encoding HA-tagged hDaxx or RGS-His_6-epitope-tagged PML, two-color confocal immunofluorescence microscopic analysis was performed. These results demonstrated co-localization of PML and hDaxx within nuclear speckles in >80% of PODs examined (Figure 6).

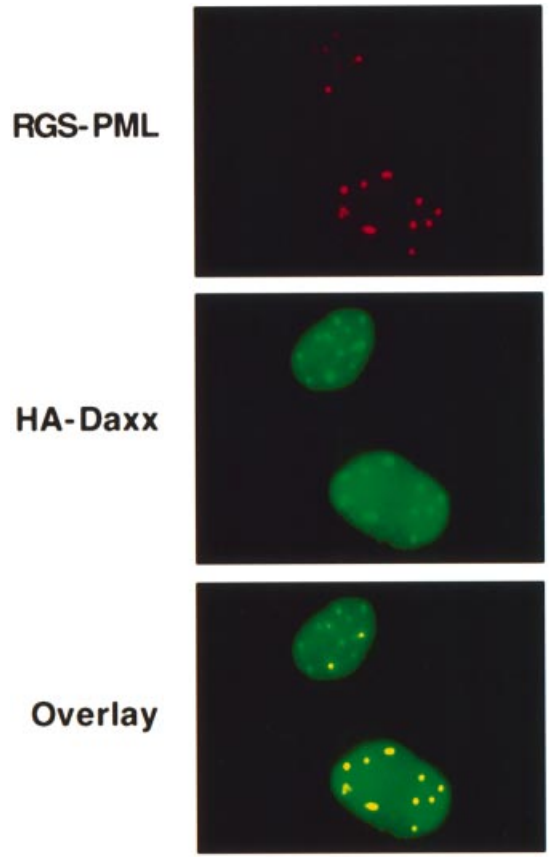

Fig. 6. hDaxx associates and co-localizes with PML. HT1080 cells stably expressing HA–Daxx were transfected with a plasmid encoding RGS-His_6-tagged PML. After fixation, cells were incubated with anti-HA rat monoclonal antibody (3F10) for the detection of Daxx and anti-RGS-His_4 mouse monoclonal antibody for the detection of PML. Antibody detection was achieved using FITC-labeled anti-rat IgG (green) and Texas Red-conjugated anti-mouse IgG (red), followed by confocal microscopy. The bottom panel shows two-color overlay results, demonstrating co-localization of the HA–Daxx and RGS-His_6-PML proteins (yellow).

Experiments performed with untransfected cells confirmed the specificity of these results (not shown).

A modulator of PODs enhances Fas-induced apoptosis

Arsenicals can restore localization of PML–RARα oncoproteins to PODs in APL cells (Zhu *et al*., 1997) and are known to enhance localization of wild-type PML and some other POD-associated proteins to these nuclear structures in non-leukemic cells (Zhu *et al*., 1997). Arsenicals such as As_2O_3 also increase apoptosis induced by overexpression of PML (Quignon *et al*., 1998). We therefore explored the effects of As_2O_3 on Fas-induced apoptosis in control and hDaxx-overexpressing cells.

Addition of 2.5 μM As_2O_3 to control-transfected HT1080 cells (Neo) augmented Fas-induced cell death (Figure 7A). The extent to which Fas-induced cell death was potentiated by As_2O_3 in control HT1080 cells was similar to that obtained by overexpressing hDaxx in these cells. Similar enhancements of Fas-induced apoptosis by As_2O_3 were observed in HT1080 and MCF7/Fas cells (not shown). More striking, however, was the effect of adding As_2O_3 to cells that overexpress hDaxx. In hDaxx-overexpressing HT1080 cells, for example, the percentage of dead cells 1 day following stimulation with anti-Fas antibody CH11 was more than double that of control-transfected cells (Figure 7A). In contrast to As_2O_3, which exhibited synergy with hDaxx, Fas-induced cell death was augmented to similar extents in Neo-control and hDaxx-overexpressing cells when cultured with other potentiators of Fas such as CHX or wortmannin (not shown).

The As_2O_3-mediated potentiation of cell death was evident over a wide range of anti-Fas antibody concentrations (Figure 7B), demonstrating that As_2O_3 did not merely shift the dose–response curve to the right, but rather increased the percentage of cells undergoing Fas-induced apoptosis at all concentrations of anti-Fas antibody used. Although it potentiated Fas-induced apoptosis, As_2O_3 did not induce cell death by itself at the concentrations employed for these experiments and it did not increase cell death induced by Fas-independent cell death stimuli such as staurosporine (not shown).

In addition to displaying synergistic effects with hDaxx in terms of cell death induction, As_2O_3 also altered the nuclear distribution of hDaxx. Localization of epitope-tagged hDaxx by immunofluorescence microscopy revealed that the fluorescence signals from nuclear speckles became more intense following As_2O_3 treatment while the diffuse nucleoplasmic fluorescence attributed to hDaxx diminished simultaneously (Figure 7C). Immunostaining using an anti-PML antiserum confirmed that these speckles represented PODs (not shown). Consistent with recent investigations of PML distribution following As_2O_3 exposure (Zhu *et al*., 1997), the number of nuclear speckles visualized by immunolocalization of either HA–hDaxx or Flag–hDaxx was decreased overall after As_2O_3 treatment, but the apparent size of individual speckles was increased. The effects of As_2O_3 on Daxx immunolocalization were dependent on the time of exposure in culture (not shown), arguing that the mere addition of As_2O_3 to cell samples does not non-specifically enhance or alter immunodetection of the HA–Daxx or Flag–Daxx proteins. These findings suggest that hDaxx, like PML, is recruited to PODs by treatment with As_2O_3, correlating with enhanced cellular sensitivity to Fas-induced apoptosis.

Loss of Fas-potentiating function of hDaxx mutants with defective POD localization

The hDaxx protein contains two candidate NLS motifs, consistent with its targeting to nuclei (Pluta *et al*., 1998). To explore the relevance of nuclear targeting of Daxx for its Fas-potentiating function, we generated hDaxx mutants in which either the proximal NLS was altered by site-directed mutagenesis, converting residues 391–395 from RKKRR to RTKSR (hDaxx-MT), or in which the distal candidate NLS was eliminated by the introduction of a stop codon at position 625, thereby generating a C-terminal truncation mutant (hDaxx-ΔC) (Figure 8A). Neither of these mutations individually prevented nuclear targeting of hDaxx (Figure 8B). Combining these mutations resulted in the appearance of some cytosolic hDaxx, but did not prevent entry of hDaxx into nuclei (not shown), suggesting that other regions of the protein are responsible for its nuclear targeting. However, because the hDaxx (ΔC) mutant failed to localize to nuclear bodies (Figure 8B), it provided an opportunity to explore the relevance of POD

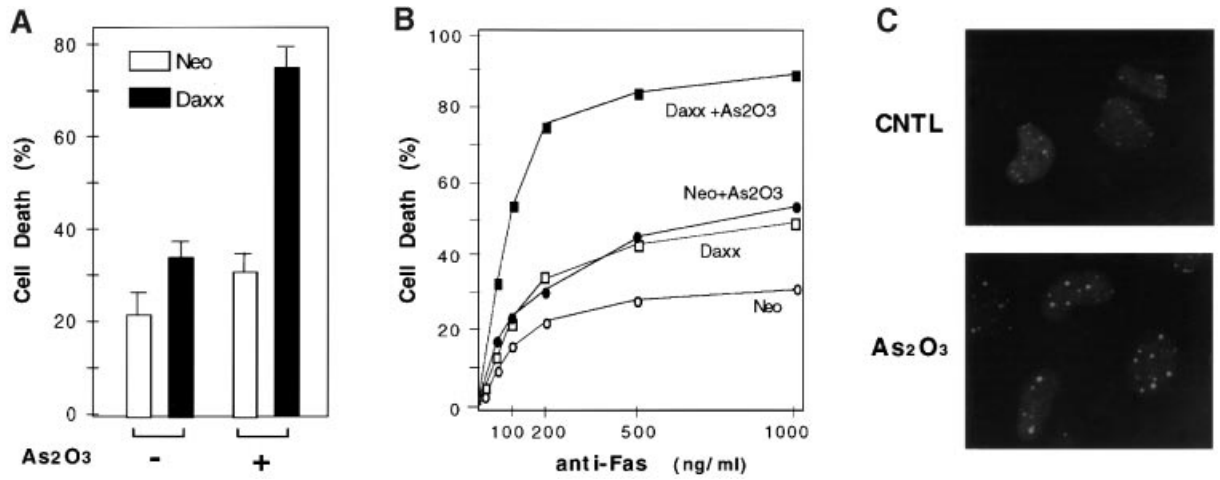

Fig. 7. Arsenic treatment sensitizes Daxx-overexpressing cells to Fas-induced cell death and promotes localization of Daxx protein to nuclear bodies. (**A**) HT1080/pcDNA3 (Neo) (white bars) and HT1080/HA–Daxx (black bars) cells were incubated with 0.2 μg/ml of anti-Fas monoclonal antibody (CH11) with or without 2.5 μM As_2O_3. After 24 h, both floating and adherent cells were recovered and subjected to Trypan Blue dye exclusion assay (mean ± SE; $n = 3$). (**B**) HT1080-Neo (circles) and HT1080-Daxx (squares) stable cell lines (1×10^6) were incubated with (black symbols) or without (white symbols) 2.5 μM As_2O_3 plus various concentrations of anti-Fas antibody. The percentage of dead cells was determined by Trypan Blue dye uptake at 24 h. (**C**) HT1080 cells stably expressing HA–Daxx were cultured without (CNTL) or with 5 μM As_2O_3 for 6 h. After fixation, permeabilized cells were incubated with anti-HA rat monoclonal antibody (3F10) followed by FITC-labeled anti-rat IgG. Similar results were obtained using HT1080 cells transfected with Flag–Daxx and stained with anti-Flag monoclonal antibody (not shown).

association to the function of hDaxx as a potentiator of Fas-induced apoptosis.

Figure 8C shows typical results for MCF7/Fas cells, which were transiently transfected with plasmids encoding wild-type (WT) hDaxx, hDaxx (MT), hDaxx (ΔC) or a control plasmid (Neo) and then stimulated ~1 day later with anti-Fas antibody. As shown, WT hDaxx and the mutant hDaxx (MT) protein that retained POD localization both enhanced Fas-induced cell death to similar extents in MCF7/Fas cells (Figure 8C; left panel). In contrast, the hDaxx (ΔC) mutant that failed to associate with PODs did not enhance Fas-induced cell death.

Similar results were obtained in HT1080 cells that had been stably transfected with plasmids encoding WT, MT or ΔC hDaxx proteins (Figure 8C; right panel). Again, cells overexpressing WT hDaxx or the hDaxx (MT) protein that localized to PODs displayed increased sensitivity to Fas-induced apoptosis. In contrast, the hDaxx (ΔC) mutant with defective POD targeting failed to increase cell death induction in response to anti-Fas antibody. Immunoblot analysis confirmed expression of the WT, MT and ΔC hDaxx proteins at comparable levels in HT1080, MCF7/Fas and other types of cells (Figure 1C; data not shown). None of the hDaxx variants tested here induced cell death in the absence of anti-Fas antibody stimulation, indicating that they do not trigger apoptosis directly (Figure 8C).

hDaxx is a modulator of transcription

Recently, it has been shown that PML interacts with the transcriptional co-activator CBP and co-localizes with nascent mRNA transcripts in PODs, suggesting that PML is a transcriptional regulator and that PODs represent a site of active gene transcription within nuclei (La Morte *et al*., 1998; Doucas *et al*., 1999). As an initial attempt to explore the possibility that hDaxx might also regulate transcription, experiments were performed in a heterologous transcriptional reporter system in which hDaxx or various fragments thereof were expressed as fusion proteins with the Gal4 DNA-binding domain (DBD) in CV1 cells. The transcriptional activity of a luciferase reporter gene plasmid containing several copies of a Gal4 binding site within its promoter was then assessed.

As shown in Figure 9, transcriptional activation of the luciferase reporter plasmid was readily detected in cells expressing the Gal4-DBD protein without hDaxx appended. In contrast, little reporter gene activity was observed when Gal4-DBD was fused with the full-length hDaxx protein, suggesting that hDaxx repressed transcription in this assay (~27-fold decrease). Unlike the Gal4-DBD fusion protein containing full-length hDaxx, a Gal4–hDaxx (ΔC) mutant lacking the C-terminal region shown to be important for localization to PODs (Figure 8) had substantially less repressive effect in this assay (only ~3-fold suppression). Interestingly, fusing the C-terminal domain of hDaxx (residues 625–740) to Gal4-DBD was sufficient to suppress transcriptional activation of the luciferase reporter plasmid (~11-fold in this assay). Thus, this C-terminal domain of hDaxx is both necessary and sufficient for modulating transcription in this system. Immunoblot analysis confirmed production of the various Gal4-DBD proteins at comparable levels (not shown).

Finally, having obtained evidence that hDaxx may function as a transcriptional modulator, we examined hDaxx-transfected cells for changes in expression at the protein or mRNA levels of several genes implicated in Fas signaling or Fas resistance, including Fas, Fas-L, Fadd, caspase-8, caspase-10, Flip and DAP3. However, no consistent difference in the expression of these genes was evident (Figure 10; data not shown).

Discussion

The mouse and human Daxx proteins reportedly bind to the death domain of Fas, and mouse Daxx regulates an apoptosis pathway involving direct interactions with the MAP3K protein Ask-1, which in turn leads to JNK

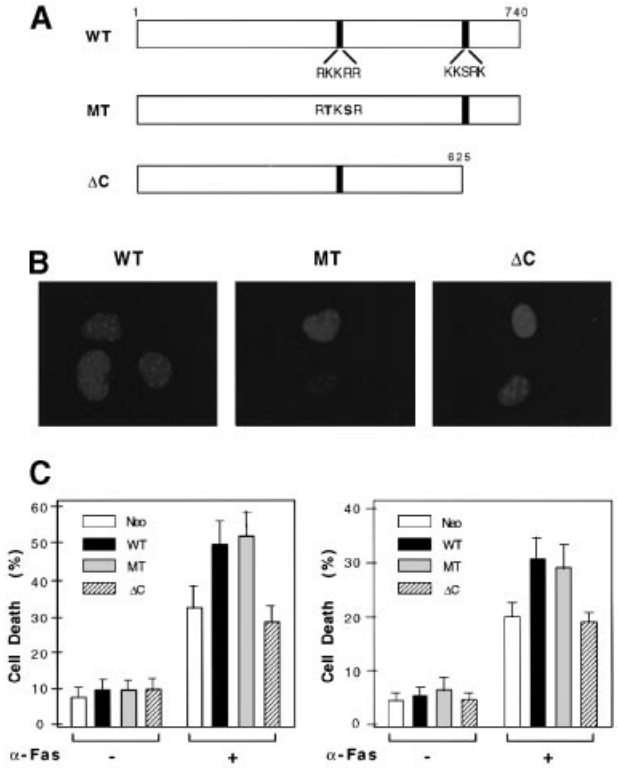

Fig. 8. The C-terminal domain of hDaxx is essential for both apoptotic activity and localization to PODs. (**A**) Schematic representation of human Daxx and its mutants. The two predicted nuclear localization signal sequences are shown (black boxes). (**B**) Immunofluorescence analysis of Daxx mutants. HT1080 cells stably expressing HA-tagged WT, MT or ΔC Daxx proteins were fixed, permeabilized and incubated with anti-HA rat monoclonal antibody (3F10) followed by FITC-labeled anti-rat IgG. (**C**) Apoptosis analysis of Daxx mutants. Left panel, MCF7/Fas cells were transiently transfected with 1 μg of pcDNA3 control plasmid or various Daxx expression plasmids. After 20 h, cells were incubated with anti-Fas antibody for 12 h and the percentage of dead cells was determined by Trypan Blue dye uptake. Right panel, HT1080 stable cell lines expressing HA–Daxx or HA–Daxx mutants were incubated with anti-Fas antibody and percent cell death was determined as above.

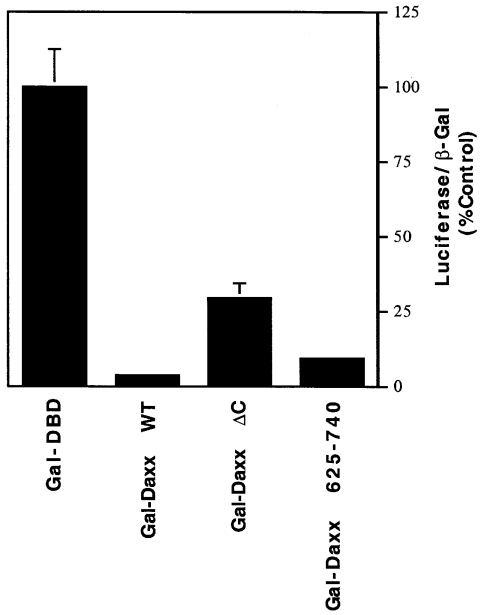

Fig. 9. hDaxx represses transcription. Plasmids (0.1 μg) encoding full-length Daxx 1–740 c DNA (WT), Daxx 1–625 (ΔC) and Daxx 625–740 fused to amino acids 1–147 of the yeast Gal4 DBD were co-transfected into CV-1 cells with 1.0 μg of the pMH100 reporter plasmid and 1.0 μg of pCMV-β-Gal. Cells were lysed 24 h later and luciferase and β-galactosidase activities were measured. Results represent relative luciferase activity normalized for β-galactosidase (mean ± SE; $n = 3$).

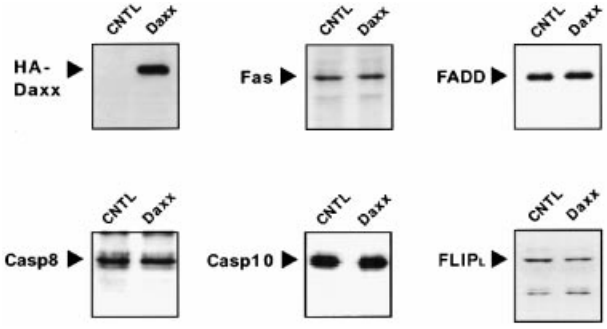

Fig. 10. Comparison of apoptosis-regulatory protein levels in control and hDaxx-overexpressing cells. Cell extracts were prepared from both HT1080 control cells and HT1080/Daxx stable cells, normalized for total protein content (50 μg) and subjected to SDS–PAGE and immunoblot analysis. Blots were probed with antibodies specific for caspase-8, caspase-10, Fadd, Fas, Flip and HA.

activation (Yang *et al*., 1997; Chang *et al*., 1998). Similar to its murine counterpart, we show here that the only known human homolog of Daxx also modulates apoptosis signaling by Fas. However, the hDaxx protein does not bind Fas and instead is found within the nucleus (Pluta *et al*., 1998), in association with PODs. Evidence supporting a functionally important role for hDaxx targeting to PODs for its Fas-potentiating activity was provided by the observations that: (i) As_2O_3, an agent that increases POD size and promotes localization of PML and of hDaxx to PODs (Zhu *et al*., 1997), demonstrated synergy with hDaxx overexpression in specifically enhancing Fas-induced apoptosis; and (ii) a mutant of hDaxx that failed to localize to PODs was unable to enhance Fas-induced apoptosis. In contrast to murine Daxx, we did not detect effects of hDaxx on JNK activation (Figure 4) and were unable to demonstrate interactions of hDaxx with Ask1 or effects of hDaxx overexpression on Ask1 kinase activity (unpublished observations). Thus, hDaxx does not appear to affect Fas signaling through effects on these protein kinases, although we cannot exclude the possibility that hDaxx might modulate them in some cellular contexts. Rather, hDaxx overexpression was associated with accelerated Fas-induced processing of the caspase-8 substrates, pro-caspase-3 and Bid, suggesting that hDaxx somehow facilitates the caspase-dependent arm of the Fas signaling pathway rather than the putative caspase-independent JNK pathway.

The association of hDaxx with nuclear PODs is intriguing, given recent evidence implicating PML in apoptosis regulation. Unlike hDaxx, where mere overexpression of the protein was insufficient to trigger apoptosis, PML overexpression has been shown to induce cell death through an undefined mechanism (Quignon *et al*., 1998). Moreover, arsenic synergizes with PML to induce cell death, similar to our observations with Fas-induced apoptosis in cells exposed to arsenic in combination with high levels of hDaxx. However, PML was reported to induce cell death even in the presence of broad-spectrum

caspase inhibitors (e.g. zVAD-fmk), which is clearly distinct from Fas-induced apoptosis in either the presence or absence of hDaxx overexpression (not shown). Thus, the mechanisms used by PML and hDaxx to modulate cell death pathways may not be identical, or the experimental systems employed here may simply have failed to reveal the full spectrum of hDaxx activities.

Mice with homozygous *pml* gene disruptions display defects in apoptosis induction by Fas and some other stimuli, including X-irradiation, TNF, interferon and ceramide (Wang *et al*., 1998b). This spectrum of apoptosis defects is broader than we observed with hDaxx, which enhanced apoptosis induced by Fas when overexpressed in cells but failed to have detectable effects on apoptosis induced by overexpression of TNFR1, DR4, DR5, Fadd, caspase-8 or caspase-10. Cells overexpressing mDaxx have also been reported to undergo increased apoptosis in response to Fas but not to TNFR1 or Fadd (Yang *et al*., 1997). Several explanations for differences in the spectrum of apoptosis stimuli affected by hDaxx and PML are possible. First, PML and Daxx may modulate an overlapping but non-identical subset of apoptosis regulatory proteins or genes. Secondly, results from gene knock-outs (e.g. *pml*) may yield more complete phenotypes than attempts at overexpression (e.g. hDaxx). Thus, in our experiments, endogenous hDaxx levels may have been limiting only for Fas-induced apoptosis, but not for cell death induced by other apoptotic stimuli.

In cells derived from $pml^{-/-}$ mice, it was observed that activation of caspase-1 and caspase-3 following X-irradiation or anti-Fas antibody stimulation was impaired (Wang *et al*., 1998b). This suggests that the PML protein is required for efficient coupling of these death stimuli to caspase activation. Similarly, we observed that hDaxx overexpression accelerated the processing of caspase-3 and of the caspase-8-substrate Bid. Although we only variably detected more rapid processing of pro-caspase-8 in Fas-stimulated cells that overexpress hDaxx, proteolytic processing of two proteins that are known to be direct substrates of caspase-8 (i.e. Bid and pro-caspase-3; Li *et al*., 1998; Luo *et al*., 1998; Stennicke *et al*., 1998) was consistently faster and more extensive. Thus, both PML and hDaxx appear to modulate steps involved in the activation of caspases, although the mechanisms responsible remain to be determined.

PODs are thought to be sites of transcriptional regulation (La Morte *et al*., 1998). We presume that hDaxx modulates pathways involved in Fas-induced apoptosis through its ability to modulate transcription, based on our findings that hDaxx localizes to PODs and that it represses transcription when fused to Gal4-DBD. Supporting this hypothesis are the observations that the C-terminal domain of hDaxx (residues 625–740) was found to be required for: (i) enhancing Fas-induced apoptosis; (ii) localization of hDaxx to PODs; and (iii) optimal activity in transcriptional repression assays. A role for hDaxx in transcriptional repression is also consistent with its reported association with a centromere-binding protein (Pluta *et al*., 1998) inasmuch as centromeres are sites of heterochromatin where gene silencing predominates (Lamond and Earnshaw, 1998). While it is possible that hDaxx may enhance rather than repress transcription in some contexts, potentiation of Fas-induced apoptosis by hDaxx overexpression was not impaired by CHX, suggesting that increased protein synthesis may not be required. Similarly, apoptosis induced by overexpression of PML does not require *de novo* transcription (Quignon *et al*., 1998). In this regard, the PML protein reportedly can either enhance or suppress transcription, depending on the cellular context (Mu *et al*., 1994; Guiochon-Mantel *et al*., 1995; Vallian *et al*., 1997; Doucas *et al*., 1999). For example, PML binds the transcriptional coactivator and histone acetyltransferase (HAT) CBP *in vitro*, promotes CBP localization to PODs, and enhances transcriptional activity of nuclear receptors such as glucocorticoid receptor (GR) and retinoid-X-receptor (RXR) in transient transfection reporter gene assays (Doucas *et al*., 1999). Conversely, fusion of PML to Gal4-DBD results in repression of transcription from Gal4-responsive reporter plasmids (Vallian *et al*., 1997), similar to our observations here with hDaxx. Thus, we consider it likely that PML and Daxx are transcriptional modulators whose effects on gene expression (up- or downregulation) may vary depending on interactions with other factors or a variety of circumstances. It remains to be determined whether hDaxx and PML interact either physically or functionally within PODs in controlling gene expression.

Interestingly, the C-terminal region of mDaxx corresponding to residues 625–740 has been reported to be necessary and sufficient for binding Fas and is required for enhancing Fas-induced apoptosis (Yang *et al*., 1997). In this report, we observed that the analogous C-terminal segment of hDaxx displayed the ability to repress transcription when linked to Gal4-DBD and was also necessary for efficient targeting of hDaxx to PODs and for potentiation of Fas-induced apoptosis. Thus, the C-terminal domain of Daxx appears to play an important role in its regulation of apoptosis pathways involving Fas. Based on our observations, we favor the hypothesis that Daxx performs its Fas-potentiating function by modulating gene expression from PODs, rather than by binding directly to Fas.

Attempts to identify target genes of hDaxx regulation demonstrated no consistent difference in the steady-state levels of several proteins or of the corresponding mRNAs encoding proteins previously implicated in induction or suppression of Fas-induced apoptosis, including Fas itself, Fas-L, Fadd, Flip, pro-caspase-8, pro-caspase-10 and DAP-3. Although unchanged in their levels, we cannot exclude the possibility that hDaxx alters the expression of other genes whose encoded proteins modulate the functions of some of the apoptosis-regulatory proteins examined here, by inducing post-translational modifications of them (Cardone *et al*., 1998; Mannick *et al*., 1999) or by altering their intracellular location through protein interactions or other mechanisms (Bennett *et al*., 1998; Perez and White, 1998; Siegel *et al*., 1998). Whatever the explanation, however, the observation that hDaxx specifically enhances apoptosis induced by Fas, but not cell death induced by overexpression of other TNF-family death receptors, Fadd or pro-caspase-8, suggests that hDaxx modulates a private pathway involved in Fas-induced apoptosis. This suggests that the actions of hDaxx map to a proximal step in Fas signaling, perhaps influencing the efficiency of receptor coupling to downstream caspases, the efficiency of release of activated caspases from receptor complexes into the cytosol or other related

steps in the immediate post-receptor events that participate in Fas-induced apoptosis.

The effects of Daxx on cell death pathways may however be cell-type specific. Thus, while a selective effect on Fas-induced apoptosis was detected in the tumor cell lines evaluated here, we cannot exclude the possibility that Daxx may modulate sensitivity to other apoptotic stimuli in other cellular contexts. For example, mice with homozygous disruption of their *Daxx* genes have recently been reported, revealing embryonic lethality in association with increased apoptosis (Michaelson *et al*., 1999). This finding supports a role for Daxx as a regulator of apoptosis, but suggests that in some cellular contexts it may suppress rather than promote cell death. Previously, it has been shown that other apoptosis regulators can either inhibit or induce cell death, depending on the particular types of cells interrogated or on the ratios of these proteins relative to other apoptosis modulators (Knudson *et al*., 1995; Chen *et al*., 1996; Middleton *et al*., 1996; Oh *et al*., 1997). In an analogous manner, the amount of Daxx produced in cells relative to other unidentified factors may similarly dictate whether a pro- versus anti-apoptotic phenotype is produced by manipulations of the levels of Daxx protein. Alternatively, because these animals produced an altered mDaxx transcript (Michaelson *et al*., 1999), they may not be entirely null for Daxx and potentially could produce fragments of the Daxx protein that display hyperapoptotic function, analogous to those previously produced experimentally (Yang *et al*., 1997). Regardless of the explanation, the findings reported here demonstrate that Daxx exhibits its apoptosis-modulatory functions from within the nucleus, probably by interacting with other proteins present in PODs.

Materials and methods

Plasmids

The cDNAs encoding C-terminal fragments (493–740) of hDaxx were obtained from a human Jurkat T-cell cDNA library, using caspase-10 as a bait during a yeast two-hybrid screen. A cDNA encoding residues 1–585 of hDaxx was generated by RT–PCR methods from Jurkat T-cell mRNA using sequences found in the DDBJ/EMBL/GenBank EST database (AA312767, AA085057) for design of amplification primers. The full-length hDaxx cDNA used for these studies was generated by PCR using the Daxx (1–585) and Daxx (493–740) cDNAs as templates and the amplification primers 5′-CCAATTCCCTATGGCCACCGCT-AA-3′ and 5′-CCCTCGAGAGGCAGTTAATCAGAGTCTGA-3′. The full-length hDaxx cDNA was cloned into the *Eco*RI and *Xho*I sites of pcDNA3-HA and pcI-FLAG plasmids (Matsuzawa *et al*., 1998). For the NLS mutant (MT), mutagenic primers were designed (5′ PCR primer: GGCGACTATGTGAGCTGAA; 3′ primer: CGAGCTCTACTCTTTG-TTCTCTCGC) and subjected to PCR, and PCR product was cloned into *Eag*I and *Sac*I sites of human Daxx cDNA. The caspase-8, caspase-10, CrmA, RAIDD-Flag, Fadd (MORT1) cDNA and Fas expression plasmids have been described previously (Boldin *et al*., 1995; Fernandes-Alnemri *et al*., 1996; Frisch *et al*., 1996; Muzio *et al*., 1996; Duan and Dixit, 1997). pMH100 is a luciferase reporter construct that contains a TK promoter and four Gal4 binding sites (Doucas *et al*., 1999). pCMX Gal DBD is a mammalian expression vector that allows for the generation of Gal-DBD fusion proteins (Chen *et al*., 1996b). Gal4-DBD fusions containing full-length Daxx and Daxx fragments were generated by PCR and cloned into the *Bam*HI site of pCMX Gal DBD C-terminal to amino acids 1–147 of the yeast Gal4 DBD.

Cell culture and transfection

Human embryonic kidney 293, 293T cells, human fibroblast GM701 cells and human fibrosarcoma HT1080 cells were grown in high-glucose Dulbecco's modified Eagle's medium containing 4.5 g/dl glucose supplemented with 10% fetal calf serum (FCS), 1 nm L-glutamine, and antibiotics. Human breast cancer MCF7/Fas cells were similarly maintained in RPMI1090 medium. For transient transfections, cells (~5×10^5) were transfected with 0.1 μg of pEGFP in combination with 0.3–0.8 μg of various plasmids using 8 μl SuperFect reagent (Qiagen) and studies were performed after 1 day. For stable transfections, HT1080 cells and MCF7/Fas cells were transfected with 2 μg of various plasmids in 60 mm dishes, using 8 μl SuperFect transfection reagent (Qiagen) and stable transfectants were selected by culturing in the presence of 1.0 mg/ml (active compound) G418 (Calbiochem). In some experiments, cells were cultured with various concentrations of anti-Fas monoclonal antibody CH11 (500 μg/ml; Medical & Biological Laboratories, Co.), etoposide (VP16), TNF-α (10 μg/ml; Genzyme), CHX (Sigma) or As_2O_3 (Sigma).

Apoptosis and cell death assays

For assessing apoptosis, floating and attached cells were collected, pooled, fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min and then stained with 10 μg/ml of DAPI. The percentage of apoptotic cells revealed by DAPI staining was determined by fluorescence microscopy, counting only the GFP-positive cells in those case where GFP was employed as a transfection marker. For assessing cell death, pooled floating and adherent cells were resuspended in PBS containing 0.2% Trypan Blue dye and the percentage of cells that failed to exclude dye was determined.

Immunoblotting

Cells were lysed in modified Laemmli buffer (60 mM Tris pH 6.8, 10% glycerol and 2% SDS). Lysates were normalized for total protein content by the bicinchoninic acid method (Pierce) and subjected to SDS–PAGE, followed by transfer to nitrocellulose filters. As described previously, blots were incubated with various antibodies directed against caspase-3 (Krajewska *et al*., 1997), caspase-8 (rabbit polyclonal raised against C-terminal peptide 458–479), caspase-10 (rabbit polyclonal raised against recombinant GST fusion), Flip (PharMingen), Bid (rabbit antiserum raised against recombinant protein), Fas (DX2) (PharMingen), Fadd (PharMingen) and α-tubulin (clone DM-1A; Sigma). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham), the blots were developed using the emission chemiluminescence (ECL) detection method with exposure to X-rays (Krajewski *et al*., 1996).

Co-immunoprecipitation

293T cells (~1×10^6) were transiently transfected with 4 μg of each expression plasmid. After 24 h, cell lysates (500 μg total protein in 1 ml) were prepared from transfected cells using IP buffer (20 mM Tris pH 7.5, 0.5% NP-40, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and incubated with 30 μl of anti-Flag antibody M2-conjugated agarose (Sigma) or with a combination of 5 μg of anti-Fas antibody (DX2) and 30 μl recombinant protein G–Sepharose 4B (Zymed) at 4°C for 8 h. Immunoprecipitates were washed with 1.5 ml IP buffer at least four times and suspended in SDS sample buffer. Immune complexes were analyzed by SDS–PAGE and immunoblotting using 0.1 μg/ml anti-HA antibody 3F10 (Boehringer Mannheim) followed by HRP-conjugated anti-rat IgG. Detection was by ECL (Amersham).

Indirect immunofluorescence microscopy

Cells (10^3) were cultured on 8-well Lab-Tek chamber slides (Nalge Nunc International) for 1–2 days. The cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate pH 7.3 containing 3% sucrose for 30 min at 4°C. After fixation, cells were rinsed and permeabilized three times for 10 min with high-salt TPBS (0.01 M sodium phosphate, 0.5 M NaCl, 0.1% Tween-20 pH 7.3) containing 0.1% Triton X-100. The permeabilized cells were blocked with PBS containing 2% FCS (30 min, 20°C) and then incubated for 18 h at 4°C with primary antibody diluted in PBS containing 0.05% Tween-20. Following this incubation, cells were rinsed three times for 10 min with high-salt TPBS at room temperature and then incubated at 8 μg/ml with fluorescein isothiocyanate (FITC)- or Texas Red-conjugated anti-rabbit, anti-rat or anti-mouse IgG (Dako) for 1 h at 20°C. Excess secondary antibody was thoroughly washed off with high-salt TPBS. The slides were then treated with Vectashield mounting medium (Vector Laboratories) and glass coverslips were applied. The stained cells were observed using a laser-scanning confocal microscope (Bio-Rad 1024MP).

Transcription repression assays

CV-1 cells were transfected in 96-well plates using DOTAP, essentially as described (Doucas *et al*., 1999). Cells were co-transfected with 1 μg

of pMH100 luciferase reporter construct, 1 μg of pCMX β-gal and 100 ng of pCMX Gal DBD, pCMX Gal Daxx 1–740, pCMX Gal Daxx 1–625 or pCMX Gal Daxx 625–740. Luciferase activity was normalized to β-gal activity to control for transfection efficiency.

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