The death domain-associated protein modulates activity of the transcription co-factor Skip/NcoA62

Jun Tang[a], Howard Y. Chang[b], Xiaolu Yang[a,*]

[a] *Abramson Family Cancer Research Institute and Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

[b] *Program in Epithelial Biology, Departments of Dermatology and Genetics, Stanford University School of Medicine, Stanford, CA 94305-5168, USA*

Received 22 February 2005; revised 30 March 2005; accepted 8 April 2005

Available online 27 April 2005

Edited by Frances Shannon

Abstract Death domain-associated protein (Daxx) regulates both transcription and apoptosis. The role of Daxx in transcription is not well understood. Here, we show that Daxx interacts with Skip/NcoA62, a transcription cofactor that modulates the activity of oncoproteins including Ski and NotchIC. Daxx strongly binds with Skip both in vitro and in mammalian cells. This interaction is mediated by the PAH2 domain of Daxx and the highly conserved SNW domain of Skip. Daxx partially co-localizes with Skip in vivo and changes the cellular distribution of Skip. In addition, Skip represses transcription when tethered to a promoter, and Daxx antagonizes this activity. Furthermore, Skip is phosphorylated at serine 224 in its SNW domain. These results suggest a novel function of Daxx in transcription regulation through alteration of the cellular localization of Skip.

Keywords: Daxx; Skip; NcoA62; Transcription; Interaction

1. Introduction

The death domain-associated protein (Daxx) regulates apoptosis as well as transcription. It was first discovered as an adapter protein that interacts with the intracellular region of the CD95 (Fas/Apo-1) death receptor and participates in CD95-mediated apoptosis through the activation of Jun NH_2-terminal kinase (JNK) [1]. Daxx interacts with a JNK kinase kinase, the apoptosis signal-regulating kinase 1 (ASK1), relieving an inhibitory intramolecular interaction between its NH_2- and COOH-terminal regions [2,3]. Daxx also associates with the cytoplasmic domain of the transforming growth factor-β (TGF-β) receptor, linking this receptor to the JNK pathway and contributing to apoptosis [4]. Daxx can be found in both the nucleus and the cytosol. However, in most cell types, Daxx is predominantly localized in the nucleus. Translocation of Daxx to the cytosol occurs upon CD95 engagement, which is enhanced by ASK1 and inhibited by the heat shock protein 27 [5–7]. In contrast, Daxx is present in the cytosol in primary splenocytes and translocates to the nucleus upon mitogenic activation, where it participates in apoptosis induction [8]. Similarly, Daxx moves from the cytosol to the nucleus during interferon-induced cell death of progenitor B cells in a Tyk2 (a nonreceptor tyrosine kinase of the Jak family)-regulated manner, which promotes apoptosis [9,10].

Nuclear Daxx primarily resides in the promyelocytic leukemia protein nuclear bodies (PML-NBs), subnuclear structures whose disruption is implicated in the pathogeneses of promyelocytic leukemia. PML-NBs may regulate a range of important cellular processes such as anti-viral responses, cellular senescence, and apoptosis [11–13]. The interaction of Daxx with the PML-NBs is mediated by PML [8,14,15], and the apoptotic function of nuclear Daxx is likely related to its PML-NB localization [8,16]. Accumulating evidence suggests that nuclear Daxx plays a role in transcriptional regulation. When tethered to DNA, as both the NH_2- and the COOH-terminal regions of Daxx are able to do, Daxx strongly represses transcription [15]. The Daxx COOH-terminal region interacts with sequence-specific transcription factors Pax3, Pax5, ETS-1, and the glucocorticoid receptor, and it either inhibits or enhances transcription in a cell- and promoter-specific manner [17–20]. The NH_2-terminal region has recently been shown to associate with ATRX, a chromatin-remodeling molecule whose mutations are associated with X-linked mental retardation and α-thalassemia syndrome (ATR-X syndrome). Daxx and ATRX form a novel chromatin-remodeling complex, with ATRX being the core ATPase and Daxx being the targeting subunit [21,22]. Despite identification of these Daxx interactors, the role of Daxx in transcription remains unclear.

We hypothesized that additional effector proteins may interact with the NH_2-terminal region of Daxx, allowing Daxx to target other general transcriptional regulators to sequence-specific DNA binding proteins and their associated promoters. We performed a yeast two-hybrid screen and identified Ski interacting protein (Skip, also known as NcoA-62) as having a strong interaction with Daxx. Skip is a nuclear protein highly conserved in diverse species, including animals, yeast, and plants [23–26], and it is essential for the survival of these organisms [23–26]. Several lines of evidence indicate that Skip is a critical transcription co-factor. First, Skip associates with the oncoprotein Ski, the cellular counterpart of v-Ski and a critical component of a histone deacetylase complex required for transcription repression by the thyroid hormone receptor [23,27]. Second, Skip enhances gene expression mediated by the vitamin D receptor and other transcription factors [24,28–30]. In addition, Skip is involved in the activation of the CBF1-repressed gene by EBNA2 (the Epstein-Barr virus encoded latency protein) and the active form of the transmembrane receptor Notch (NotchIC) [31,32]. In the case of

*Corresponding author.
E-mail address: xyang@mail.med.upenn.edu (X. Yang).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.04.029

NotchIC, Skip binds to the DNA-specific repressor CBF1 in resting cells and facilitates targeting of the SMRT/HDAC co-repressor complex to CBF1. Upon stimulation of the Notch pathway, Skip promotes tethering of the transcription activator NotchIC to CBF-1 [32]. Thus, Skip serves as a docking site for both transcription co-activators and co-repressors. Furthermore, the *Drosophila* Skip homolog, Bx42, is associated with chromatin in transcriptionally active puffs of salivary glands and is involved in ecdysone-stimulated gene expression [25]. A recent study showed that knocking-down of Skip in *Caenorhabditis elegans* results in embryonic arrest similar to that caused by the inhibition of RNA polymerase II [33], suggesting that Skip may be an essential component of various RNA polymerase II transcription complexes. However, the mechanism that regulates Skip function is not well understood.

In this study, we show that Daxx and Skip associate with each other in mammalian cells. This interaction is mediated by the PAH2 domain of Daxx and the highly conserved SNW domain of Skip. We show that Daxx modulates Skip localization and its transcription activity. In addition, we demonstrate that Skip is phosphorylated on a serine residue within the SNW domain. These results suggest that Daxx regulates Skip's function through alteration of its cellular localization and reveals a novel function of nuclear Daxx in transcription regulation.

2. Materials and methods

2.1. Yeast two-hybrid screen

The NH_2-terminal region of murine Daxx (containing amino acids 1–501) was fused to the Gal4 DNA binding domain (Gal-DBD) in the yeast expression plasmid pASII [34]. The resulting plasmid, Gal-DaxxN was co-transformed with a library of fusions between the Gal4 activation domain and cDNAs from murine T cells (CLONTECH) into a reporter yeast strain, Y190, which harbored the *lacZ* and *his3* genes under the control of a Gal promoter. Transformants were grown on plates selective for both plasmids and His prototrophy. Positive clones were isolated and further tested for the strength of interaction by filter lift assay of β-galactosidase activity [35]. The library plasmids from the strong interacting clones were isolated and re-transformed back into the reporter strain to test for interaction with Daxx as well as with various un-related control baits. The cDNA inserts in the library plasmids that encode proteins specifically interacting with Daxx were subsequently analyzed by sequencing.

2.2. Plasmids

Human Skip was cloned from a lymph node cDNA library (CLONTECH) by PCR using a forward primer 5′ CGC**GGATCC**ATGGCGCTCACCAGCTT with a *Bam*HI site (in bold) and a reverse primer 5′ CGC**GTCGAC**TTCCTTCCTCCTCTT with a *Sal*I site (in bold). The PCR product was subcloned into pRK5 with either an NH_2-terminal HA or FLAG tag. All Skip deletion and fragments, as well as Daxx (260–740) and Daxx (157–260), were generated by PCR and cloned into pRK5 with an NH_2-terminal FLAG tag. Skip and its deletion fragments were also COOH-terminally fused to green fluorescence protein (GFP) in pEGFP-C1 (CLONTECH) and to the Gal4 DNA-binding domain in pRK5. Point mutations within the SNW domain were generated by overlap PCR. All plasmids were confirmed by sequencing. The other Daxx expression plasmids used in this study were previously described [21].

2.3. Immunofluorescence

Immunofluorescence analysis was performed essentially as previously described [21,36]. Briefly, MCF7, U2OS, and 293T cells seeded on coverslips in 24-well plates were transfected on the second day with plasmids encoding Skip-GFP and HA/FLAG-Daxx using Superfect (Qiagen) or lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and the calcium phosphate precipitation method, respectively. Twenty-four hours later, the cells were washed twice with cold PBS, fixed with 2% paraformaldehyde and permeablized in 0.2% Triton in PBS at 4 °C for 10 min. For immunostaining, MCF7 and U2OS cells were incubated with an anti-Daxx (M-112, Santa Cruz Biotechnology), followed by a Texas-red conjugated secondary antibody. Cells were dehydrated with ethanol, and mounted with Vectashield mounting medium (Vector laboratories). Images were recorded by a BioRad 1024-ES confocal laser-scanning microscope or an epi-fluorescence microscope.

2.4. Immunoprecipitation, SDS–PAGE and Western blotting

293T cells grown in 60 mm plates were transfected with the indicated plasmids (5 μg each). Twenty-four hours later, the cells were washed with cold PBS twice and lysed in 500 μl of lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 mM PMSF and aprotinin) for 15 min on ice. Cell lysates were immunoprecipitated with anti-FLAG M2 beads. After being extensively washed, the precipitated immuno-complex was resolved by SDS–PAGE and analyzed by immunoblotting with the indicated antibodies and ECL reagents. To generate recombinant FLAG-Daxx, FLAG-Daxx was transiently overexpressed in 293T cells and purified on M2 beads. The non-specific proteins were removed though sequential washes with lysis buffers containing 150 mM KCl, 0.5 M KCl and 1 M KCl. The same procedure was performed to the control, in which FLAG/pRK5 was used for transfection. HA-Skip was in vitro translated and labeled with ^{35}S-methionine using a coupled in vitro transcription/translation system (Promega). ^{35}S-Skip was incubated with immobilized FLAG-Daxx in lysis buffer at 4 °C for 2 h of incubation. After extensive wishing, the bound proteins were analyzed by SDS–PAGE and autoradiography.

2.5. In vivo labeling of Skip with ^{32}Pi

Potential phosphorylation sites on Skip were analyzed using the NetPhos program (www.cbs.dtu.dk/services/NetPhos) [37]. In vivo labeling was performed as described with modifications [38]. 293T cells grown in a 6-well plate were transfected with FLAG-Skip, FLAG-SkipM and their corresponding Ser224-to-Ala mutants. Twenty-four hours later, the cells were washed once with phosphate-free DMEM containing 10% dialyzed fetal calf serum. The cells were pre-incubated in the same medium for 2 h before adding ^{32}Pi (200 μCi/ml; Amersham Pharmacia Biotech). After two hours of labeling at 37 °C, the cells were washed with cold PBS twice and lysed in lysis buffer. FLAG-tagged proteins were immunoprecipitated with M2 beads, and the labeled proteins were analyzed by SDS–PAGE followed by autoradiography.

2.6. Reporter assay

293T cells grown in 24-well plates were transfected with the indicated plasmids, together with 0.5 μg of 5Xgal-TK-luciferase reporter gene plasmid [39] and 50 ng of pCMV-β-galactosidase. Empty vector pRK5 was added to keep the total amount of DNA transfected constant. Twenty-four hours later, the cells were lysed, and the luciferase activity was measured using a firefly luciferase system (Promega), according to the manufacturer's instructions. The activity of β-galactosidase co-transfected was also measured to normalize luciferase activity. The expression levels of Gal-DBD and Gal-Skip in whole cell lysates were examined by Western blotting with an anti-Gal-DBD antibody (Santa Cruz).

3. Results

3.1. Interaction of Daxx and Skip

To better understand the function of Daxx and in particular, its NH_2-terminal region, we performed a yeast two-hybrid screen using the murine Daxx NH_2-terminal region (amino acids 1–501) as bait. A fusion of this region with Gal4 DNA-binding domain was transformed into a reporter yeast strain together with a library of fusions of the Gal4 transcription activation domain and cDNA from murine T cells. The positive clones were selected based on both the expression of