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Apoptosis Signal-regulating Kinase 1 Controls the Proapoptotic Function of Death-associated Protein (Daxx) in the Cytoplasm*

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Although Daxx (death-associated protein) was first reported to mediate the apoptotic signal from Fas to JNK in the cytoplasm, other data suggested that Daxx is mainly located in the nucleus as a transcriptional regulator. Here, we demonstrated that cellular localization of Daxx could be determined by the relative concentration of a proapoptotic kinase, apoptosis signal-regulating kinase 1 (ASK1) by using immunofluorescence and transcriptional reporter assay. ASK1 sequestered Daxx in the cytoplasm and inhibited the repressive activity of Daxx in transcription. In addition, Daxx was bound to the activated Fas only in the presence of ASK1, accelerating the Fas-mediated apoptosis. These results suggest that Daxx requires ASK1 for its cytoplasmic localization and Fas-mediated signaling. Taken together, we could conclude that ASK1 controls the dual function of Daxx as a transcriptional repressor in the nucleus and as a proapoptotic signal mediator in the cytoplasm.

Fas ligand is known to trigger apoptosis by binding to a specific receptor, Fas, which is a member of the tumor necrosis factor receptor family (1). Upon cellular activation by Fas ligand, the ligated Fas forms death-induced signal complex (DISC)[1] composed of Fas, Fas-associated death domain protein (FADD), and caspase-8 (2, 3). Recruitment of procaspase-8 to DISC leads to its proteolytic activation initiating a cascade of caspase activation that finally induces apoptosis. The activated Casp-8 stimulates proteolytic cleavage of Bcl-2 interacting protein (BID) that initiates cytochrome *c* release in the mitochondria (4). The release of cytochrome *c* triggers the formation of a complex containing Apaf1 and procaspase-9, which is then autocleaved to process the downstream effector procaspases such as caspase-3 (5). The processing of these caspases is followed by the cleavage of apoptotic substrates, leading to the disruption of important cellular processes, changes in cellular and nuclear morphology, and ultimately to cell death (2, 3).

In addition to caspase activation cascade, Fas ligation initiates the activation of c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) (6, 7). After Fas ligation, Fas recruits an adaptor protein called Daxx that interacts with apoptosis signal-regulating kinase 1 (ASK1) activating JNK/SAPK and p38 MAPK by phosphorylation (8, 9). The death domain of Fas interacts with Daxx, not competing with FADD (10). Since overexpression of Daxx sensitizes the Fas-mediated apoptosis, it is tempting to speculate that Daxx is an adaptor protein for Fas-mediated apoptosis (6). However, the role of Daxx as an adaptor linking ASK1 to Fas has been challenged because Daxx has not been localized in the cytoplasm but detected mainly in the nucleus, interacting with nuclear proteins such as centromeric protein (CENP-C), Pax3, and promyelocytic leukemia protein (PML) (11–15). The nuclear Daxx represses transcription possibly by recruiting histone deacetylase (11). It is finally thought that the Daxx-ASK1-JNK pathway would not be essential for Fas-mediated apoptosis because the Daxx-disrupted mice are embryonic lethal with extensive apoptosis (16), and JNK1/2-disrupted mice show no inhibition of Fas-mediated apoptosis (17). Despite the results from Daxx-disrupted mice, Daxx might be an important proapoptotic molecule because it induces cell cycle arrest and cell death and sensitizes Fas-mediated apoptosis (12, 18).

We suspected that Daxx might play a diverse role in apoptosis depending on its cellular localization and cell type. To solve the seemingly paradoxical and discrepant results for the role of Daxx, we investigated whether the cellular localization and function of Daxx can be controlled by its interacting kinase, ASK1. The data of this work showed how the cellular localization of Daxx may be controlled and thus finally give a clear solution to the controversy on the cellular location and function of Daxx.

EXPERIMENTAL PROCEDURES

Cell Cultures and Materials—Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml penicillin and streptomycin in a 5% CO_2 incubator. Anti-hemagglutinin (HA), -ASK1, -Daxx, and -Fas antibodies for immunoblotting and immunoprecipitation were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-JNK/SAPK antibody was from New England Biolabs. Anti-Fas antibody for Fas ligation was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Plasmids for pcDNA-Myc-Daxx, and -Fas were generous gifts from Dr. D. Baltimore (California Institute of Technology). Plasmids for pcDNA-HA-ASK1 and -FLAG-ASK1(K709R) were from Dr. H. Ichijo (Tokyo Medical and Dental University). Gal4-tk-luciferase and Gal4-Daxx were generous gifts from Dr. R. Evans (Salk Lake Institute), and tk-β-galactosidase was from Dr. D. Moore (Baylor University Medical School).

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[1] The abbreviations used are: DISC, death-induced signal complex; FADD, Fas-associated death domain protein; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; Daxx, death-associated protein; ASK1, apoptosis signal-regulating kinase 1; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; PML, promyelocytic leukemia protein; PODs, PML oncogenic domains.

DNA Transfection and Immunoprecipitation—100-mm dishes of 293 cells were transfected with the indicated plasmids using Geneporter (Gene Therapy Systems) according to the manufacturer's protocol. Twenty-four h after transfection, cells were lysed with 20 mM Tris-HCl (pH 7.5) buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 12 mM β-glycerol phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin. ASK1 or Fas in the cell lysate was reacted with the indicated primary antibody (5 μg) at 4 °C for 1 h. After addition of 50 μl of protein A-agarose, the mixture was incubated at 4 °C for an additional 4 h. The beads were washed four times with 20 mM Tris-HCl (pH 7.5) buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. The precipitated proteins were resolved on 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The immunoprecipitates were analyzed by immunoblotting with indicated primary antibodies.

ASK1 Kinase Assay and Reporter Gene Assay—The kinase activity of ASK1 was determined as described previously (7). For Gal4-tk-luciferase reporter gene assay, 293 cells grown in 24-well plates were cotransfected with the indicated plasmids, Gal4-tk-luciferase (50 ng), and tk-β-galactosidase (50 ng). After an incubation of 24 h, cells were lysed in 150 μl of reporter lysis buffer (Promega), and 20 μl of the lysate was assayed in a Luminometer (MicrolumatPlus, EG&G) with a luciferase and β-galactosidase assay system (Promega).

Immunofluorescence—Cellular localizations of Daxx and HA-ASK1 were investigated using confocal immunofluorescence microscopy (μ Radiance, Bio-Rad). The cells were grown to about 60% confluency on 5 × 5-mm coverslips and then transfected for 24 h with the indicated plasmids. The immunofluorescence procedure was previously described (19).

Apoptosis Assay—293 cells grown in 6-well plate were transiently transfected with the indicated plasmids and 0.5 μg of pcDNA-EGFP. Total amount of the transfected DNA was adjusted to be the same with pcDNA3. The transfected cells were fixed with 3% paraformaldehyde with 4,6-diamidino-2-phenylindole 24 h after transfection, and then the cell death was determined by counting the apoptotic nuclei using fluorescence microscopy.

RESULTS

ASK1 Determines the Cytoplasmic Localization of Daxx—Endo- and exogenous Daxx has been found in the nucleus, especially in the discrete nuclear structures known as PML oncogenic domains (PODs) (11, 12, 14). Since Daxx induces apoptosis only when it is localized to PODs, it is thought that Daxx would mediate apoptosis in the nucleus (14). It associates, however, with membrane-bound Fas and cytoplasmic ASK1 to transduce Fas-induced apoptosis signal (6, 8). In this case, Daxx should be localized in the cytoplasm. To address this paradoxical issue, we reinvestigated subcellular localization of Daxx by immunostaining with anti-Daxx antibody after transient expression of Daxx alone or both of Daxx and HA-ASK1 in 293 cells. Daxx was predominantly found in the nucleus with a diffused pattern when only Daxx was expressed. The diffused staining pattern instead of speckled nuclear pattern in the nucleus was previously observed in 293 cells (12). However, Daxx localization was changed to the cytoplasm when Daxx and ASK1 were co-overexpressed, suggesting that ASK1 could trap Daxx in the cytoplasm. On the contrary, the cytoplasmic localization of ASK1 was not affected by the expression of Daxx (Fig. 1*A*).

Since Torii *et al.* (12) were unable to demonstrate the interaction of Daxx with ASK1 and did not detect the effect of Daxx on the JNK activation or on the ASK1 kinase activity in 293 cells, we reinvestigated the molecular interaction of Daxx and ASK1. HA-tagged ASK1 was co-expressed with Daxx in 293 cells and immunoprecipitated with anti-HA or anti-Daxx antibodies. The immune complexes were subjected to immunoblotting with anti-Daxx or anti-HA antibodies. As shown in Fig. 1*B*, ASK1 was associated with Daxx. The molecular interaction between ASK1 and Daxx was specific because the interaction was not detected by immunoprecipitation with mock IgG antibody. To investigate the effect of Daxx on the ASK1 activity, immunoprecipitates with anti-HA antibody were subjected to a kinase assay using myelin basic protein as a substrate of ASK1. ASK1 was strongly activated by Daxx (Fig. 1*C*), supporting the previous results shown by Chang *et al.* (8).

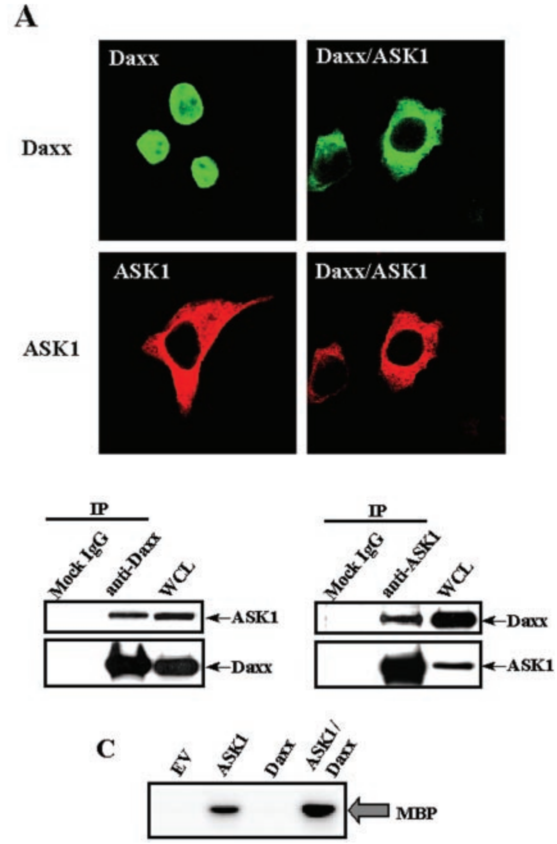

FIG. 1. *A,* ASK1 determines the cytoplasmic localization of Daxx. 293 cells in 100-mm dishes were transiently transfected with plasmids for Daxx alone (2 μg), HA-ASK1 alone (2 μg), or both of Daxx and HA-ASK1 (2 μg, respectively). The subcellular localization of Daxx and ASK1 was determined by immunostaining with anti-Daxx and anti-HA antibodies. *B,* Daxx interacts with ASK1. The plasmids expressing Daxx (2 μg) and HA-ASK1 (0.5 μg) were co-transfected into 293 cells. The proteins extracted from the transfectants were immunoprecipitated with anti-HA or -Daxx antibodies and immunoblotted with anti-Daxx or -HA antibodies. *C,* Daxx activates ASK1. Different combinations of Daxx and ASK1 were expressed in 293 cells. HA-ASK1 was precipitated with anti-HA antibody, and the kinase activity of the immunoprecipitated ASK1 was determined using myelin basic protein (*MBP*) as an exogenous substrate in the presence of [γ-^{32}P]ATP. The phosphorylated MBP was visualized by autoradiography. *EV*, *IP*, and *WCL* indicate empty vector (pcDNA3.1), immunoprecipitation, and whole cell lysate, respectively.

ASK1 Inhibits the Repression of Basal Transcription by Daxx—Daxx is known to control basal transcription (11, 13). Thus the repression of basal transcription by Daxx could be abolished if expression of ASK1 is increased because Daxx bound to ASK1 would be sequestered in the cytoplasm. To explore this possibility, we tested whether ASK1 regulates transcriptional control by Daxx. Expression of the Gal4DBD (DNA-binding domain) fused to the full-length Daxx (Gal4-Daxx) strongly inhibited the basal transcription of the Gal4-tk-luciferase reporter in 293 cells (Fig. 2). However, the repressive effect of Gal4-Daxx was abolished by the co-expression of ASK1. Thus, this result further supports the cytoplasmic interaction of ASK1 and Daxx observed by immunostaining (Fig. 1*A*).

Daxx Is Recruited to Fas When ASK1 Is Co-overexpressed—Daxx has been demonstrated to be a mediator recruiting ASK1 to Fas after Fas ligation, but the interaction between Daxx and Fas has not been observed in the cells overexpressing Daxx and

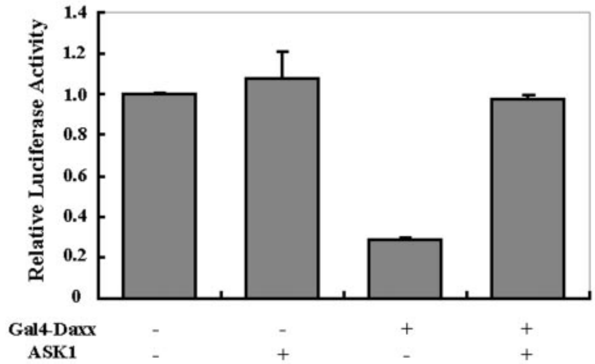

FIG. 2. **ASK1 relieves the transcriptional repression by Daxx.** Gal4-Daxx (100 ng) and ASK1 (10 ng) were co-expressed in 293 cells of 24-well plates along with Gal4-tk-luciferase (50 ng) and tk-β-galactosidase (50 ng). The total amount of the transfected DNA was adjusted to be the same with pcDNA3. Twenty four h after transfection, the activities of luciferase and β-galactosidase were determined as described under "Experimental Procedures." Luciferase activity from each transfected cell was normalized with the β-galactosidase activity.

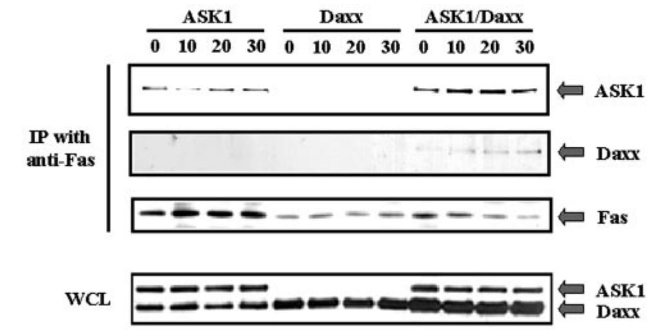

FIG. 3. **ASK1 aids the recruitment of Daxx to Fas.** The plasmids of HA-ASK1 alone (1 μg), Daxx alone (1 μg), or both of HA-ASK1 and Daxx (1 μg, respectively) were transfected into 293 cells grown in 100-mm dishes with a plasmid of Fas (0.5 μg). Twenty four h after transfection, the cells were activated for 0, 10, 20, and 30 min with anti-Fas antibody (1 μg/ml). The whole cell lysate was immunoprecipitated with anti-Fas antibody, and then the immune complex was analyzed by immunoblotting with anti-HA, Daxx, or Fas antibody. *WCL* indicates whole cell lysates; *IP* indicates immunoprecipitation.

Fas (12). Based on the data above, we thought that Daxx is not available for the association with Fas if it is located in the nucleus. Daxx could interact, however, with Fas if it is trapped in the cytoplasm associated with ASK1. To test this hypothesis, we transfected 293 cells with Daxx alone, ASK1 alone, or both of Daxx and ASK1 and investigated the molecular interaction of Fas and Daxx after Fas ligation. As shown in Fig. 3, Daxx was recruited to Fas in the cells co-expressing both of Daxx and ASK1 whereas it was not in the cells expressing either one of the two proteins, suggesting that the molecular interaction between Daxx and Fas requires ASK1.

We then tested the role of Daxx in recruiting ASK1 to Fas after Fas ligation. As shown in Fig. 3, more ASK1 was recruited to Fas after Fas ligation in the cells with Daxx and ASK1 than in the cells with ASK1 alone, implying that Daxx is a mediator for helping the recruitment of ASK1 to Fas after Fas ligation.

Co-expression of ASK1 and Daxx Accelerates Fas-mediated Apoptosis—Since cytoplasmic Daxx and ASK1 are recruited to Fas after Fas ligation, it is likely that co-overexpression of Daxx and ASK1 accelerates Fas-mediated apoptosis. To address this issue, we transiently expressed Daxx and ASK1 with different combinations into 293 cells and treated them with anti-Fas antibody to induce apoptosis. As shown in Fig. 4, Daxx or ASK1 alone did not increase the Fas-mediated apoptosis compared with control. Unlike other report showing that Daxx increases Fas-mediated apoptosis in HT1080 (12), apoptosis of 293 cells was not increased by the expression of Daxx alone. The discrepant results could reflect that apoptosis depends on the cell type and culture condition. In this regard, it is worth noting that difference in the nuclear localization of Daxx in 293 cells (Fig. 1*A*) from those reported in other cell types (12, 15). Since the localization of Daxx into PODs is required for Daxx-mediated apoptosis (14) and Daxx was not found in the PODs in the case of 293 cells, Daxx alone could not accelerate the Fas-mediated apoptosis of 293 cells. On the contrary to the effect of Daxx or ASK1 on the Fas-mediated apoptosis, co-expression of Daxx and ASK1 increased the apoptosis, suggesting that Daxx requires ASK1 for cytoplasmic localization and acceleration of the Fas-mediated apoptosis.

We also showed that ASK1 was necessary for the Fas-mediated apoptosis by transient expression of dominant negative ASK1, ASK1(K709R) (Fig. 4), consistent with previous works (8). Since ASK1(K709R) completely abolished Fas-mediated apoptosis, it is possible that ASK1(K709R) may be defective in the interaction with Daxx and thus can not control the cyto-

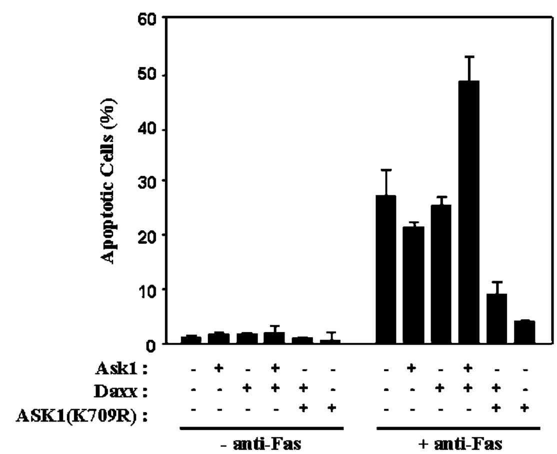

FIG. 4. **Fas-mediated apoptosis is accelerated in cells overexpressing both of Daxx and ASK1.** HA-ASK1 alone (0.5 μg), Daxx alone (0.5 μg), or both of them (0.5 μg, respectively) were transiently expressed 293 cells in 6-well plates along with 0.5 μg of pcDNA-EGFP. Twenty four h after transfection, the cells were treated with anti-Fas antibody (1 μg/ml) for 24 h. Fas-mediated apoptosis was monitored by counting apoptotic cells.

plasmic localization of Daxx. To address this issue, we investigated the localization of Daxx after transient expression of Daxx and ASK1(K709R). As shown in Fig. 5*A*, Daxx was found in the cytoplasm when it was co-expressed with ASK1(K709R), suggesting that ASK1(K709R) could also recruit Daxx to the cytoplasm like its wild type. We also tested whether Daxx is recruited to the activated Fas in the presence of ASK1(K709R). Fig. 5*B* demonstrated that Daxx and ASK1(K709R) were recruited to Fas. With all of these data, we conclude that ASK1(K709R) can still control cytoplasmic localization of Daxx and then be recruited to Fas with Daxx. Thus, the inactivity of ASK1(K709R) results from its inability to activate its downstream effector molecules.

Taken together, we concluded that Daxx requires ASK1 for its cytoplasmic localization and the molecular interaction with the activated Fas to mediate apoptosis signal. Since ASK1 also prevents the transcriptional role of Daxx (Fig. 2), ASK1 could switch the function of Daxx from the regulation of nuclear transcription to the cytoplasmic apoptosis.

DISCUSSION

Even though the Daxx-disrupted mice show an apoptosis in embryonic stages, arguing against a role for Daxx in promoting

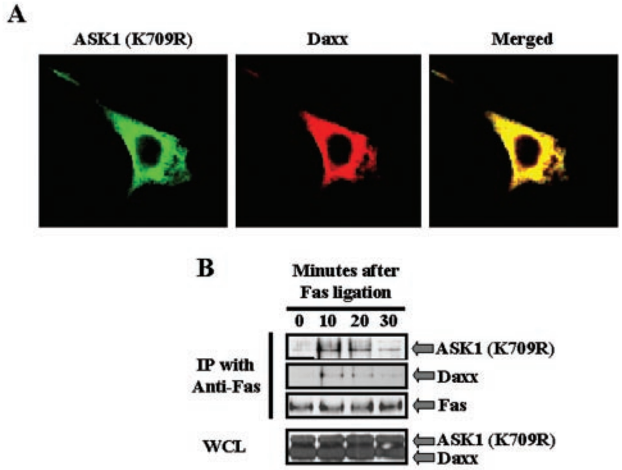

FIG. 5. *A,* Daxx is localized in the cytoplasm when it is co-expressed with a dominant negative form of ASK1, ASK1(K709R). 293 cells in 100-mm dishes were transiently transfected with plasmids for both of Daxx and ASK1(K709R) (2 μg, respectively). The subcellular localization of Daxx and ASK1 was determined by immunostaining with anti-Daxx and anti-ASK1 antibodies. *B,* Daxx is still recruited to Fas after Fas ligation when it is co-expressed with ASK1(K709R). The plasmids of Fas (1 μg), ASK1 (1 μg), and Daxx (1 μg) were transfected into 293 cells grown in 100-mm dishes. Twenty four h after transfection, the cells were activated for 0, 10, 20, and 30 min with anti-Fas antibody (1 μg/ml). The whole cell lysate was immunoprecipitated with anti-Fas antibody, and then the immune complex was analyzed by immunoblotting with anti-ASK1, Daxx, or Fas antibody. *WCL* indicates whole cell lysates; *IP* indicates immunoprecipitation.

Fas-induced cell death and suggesting that Daxx either directly or indirectly suppresses apoptosis in early embryo (16), many other groups showed that Daxx is really involved in apoptosis induction. For example, transient expression of Daxx increases Fas-induced apoptosis in 293, HeLa, L929, and HT1080 (6, 12), and a dominant negative form of Daxx (DaxxC) abrogates Fas-induced apoptosis (12, 20). Furthermore, Daxx up-regulated by interferon could be a major protein to induce apoptosis because Daxx antisense oligonucleotides rescue the interferon-treated pro-B cells from apoptosis (18). All of these data demonstrated the significant role of Daxx in apoptosis induction, and our data also demonstrated that co-expression of ASK1 with Daxx augmented this process in 293 cells (Fig. 4), indicating their functional linkage.

Since Daxx was shown to interact with diverse nuclear proteins such as PML, CENP-C, and Pax-3 (11–15), the physiological role of Daxx would be more complex than what we currently know. That is why it is so important how the cellular localization and function of Daxx can be controlled and we have clearly shown that Daxx can be trapped by ASK1 in the cytoplasm to mediate Fas-induced apoptosis (Fig. 1).

To mediate Fas signaling, Daxx should be in the cytoplasm. Our data demonstrated that ASK1 controls the cytoplasmic localization of Daxx (Fig. 1). Recruitment of Daxx to Fas and Fas-mediated apoptosis is accelerated only when Daxx is in the cytoplasm with the help of ASK1 (Figs. 3 and 4). These whole data clearly show that the cytoplasmic localization of Daxx is necessary for Fas-mediated apoptosis. Landry and co-workers also showed that Daxx could be translocated to the cytoplasm from the nucleus after Fas ligation (20). When translocation of Daxx to the cytoplasm is inhibited by Hsp27, Fas-mediated apoptosis is decreased, suggesting that cytoplasmic retention of Daxx is important for Fas-mediated apoptosis.

The expression level of ASK1 is varied spatiotemporally in developing mouse (21). For instance, ASK1 is highly expressed in suprabasal layer of epidermis and hypertrophic region of cartilage primordium of nucleus pulposus and vertebrate body, in which apoptotic cell death is implicated for the renewal of developing skin and the remodeling of cartilage and bone, respectively (21). In addition, ASK1 is up-regulated during keratinocyte differentiation (22). The variation of the ASK1 level in specific developmental stages and tissues may play an important role in controlling the cellular function of Daxx.

ASK1 plays a pivotal role in apoptosis because its overexpression induces apoptosis and its kinase-inactive mutant prevents the TNF-, Fas-, and Daxx-meditated apoptosis (8, 9). ASK1 is negatively regulated by glutaminyl-tRNA synthetase, thioredoxin, glutathione *S*-transferase Mu, 14–3-3, Akt, p21Cip1/WAF1, or HIV-1 Nef (7, 23–28), implying that ASK1 is in a central position for apoptosis signaling. Here we added one more function of ASK1 in regard to the interaction with Daxx. The role of ASK1 shown here clarified the localization and function of Daxx that remained controversial.

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