Role of the ASK1-SEK1-JNK1-HIPK1 Signal in Daxx Trafficking and ASK1 Oligomerization*

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Overexpression of JNK binding domain inhibited glucose deprivation-induced JNK1 activation, relocalization of Daxx from the nucleus to the cytoplasm, and apoptosis signal-regulating kinase 1 (ASK1) oligomerization in human prostate adenocarcinoma DU-145 cells. However, SB203580, a p38 inhibitor, did not prevent relocalization of Daxx and oligomerization of ASK1 during glucose deprivation. Studies from in vivo labeling and immune complex kinase assay demonstrated that phosphorylation of Daxx occurred during glucose deprivation, and its phosphorylation was mediated through the ASK1-SEK1-JNK1-HIPK1 signal transduction pathway. Data from immunofluorescence staining and protein interaction assay suggest that phosphorylated Daxx may be translocated to the cytoplasm, bind to ASK1, and subsequently lead to ASK1 oligomerization. Mutation of Daxx Ser667 to Ala results in suppression of Daxx relocalization during glucose deprivation, suggesting that Ser667 residue plays an important role in the relocalization of Daxx. Unlike wild-type Daxx, a Daxx deletion mutant (amino acids 501–625) mainly localized to the cytoplasm, where it associated with ASK1, activated JNK1, and induced ASK1 oligomerization without glucose deprivation. Taken together, these results show that glucose deprivation activates the ASK1-SEK1-JNK1-HIPK1 pathway, and the activated HIPK1 is probably involved in the relocalization of Daxx from the nucleus to the cytoplasm. The relocalized Daxx may play an important role in glucose deprivation-induced ASK1 oligomerization.

We previously observed that glucose deprivation increases the intracellular levels of hydroperoxide and oxidized glutathione (1). Our recent studies have shown that increases in steady-state levels of hydrogen peroxide and glutathione disulfide are sensed through thioredoxin (TRX)1 and glutaredoxin (GRX) and subsequently activate the ASK1-MEK-MAPK signal transduction pathway (2–4). TRX and GRX appear to act as physiological inhibitors of ASK1 by associating with the N-terminal and C-terminal portion of ASK1, respectively, and inhibiting ASK1 kinase activity (2, 5). TRX and GRX contain two redox-active half-cystine residues, -Cys-Gly-Pro-Cys- or -Cys-Pro-Tyr-Cys-, in an active catalytic center (5–8). These sensor molecules may be converted to the intramolecular disulfide form of TRX-(S-S) and GRX-(S-S) during glucose deprivation. The oxidized form of TRX and GRX dissociates from ASK1 and consequently activates ASK1 (2, 3, 5). Recently, we observed that release of TRX and GRX from ASK1 occurs with different mechanisms: the glutathione-dependent GRX-ASK1 pathway and the glutathione-independent TRX-ASK1 pathway (4). Disassociation of either regulator from ASK1 is sufficient for ASK1 activation (4).

ASK1 is a member of the mitogen-activated protein kinase kinase kinase family that activates the JNK and p38 pathways by directly phosphorylating and thereby activating their respective mitogen-activated protein kinase kinases, MKK4/SEK1/MKK7 and MKK3/MKK6 (9). ASK1 is activated by oxidative stress (2, 5), TNF-α (10, 11), Fas ligand (12, 13), and endoplasmic reticulum stress (14). Previous studies have shown that TNF-α activates ASK1 via TRAF2, a member of the TNF-receptor-associated factor (TRAF) family (10, 11), whereas Fas ligand activates ASK1 via Daxx (12, 13). Liu et al. (11) reported that TRAF2 activates ASK1 by enhancing and stabilizing the oligomerization of ASK1. Chang et al. (12) observed that Daxx activates ASK1 by displacing an inhibitory intramolecular interaction between the NH2 and COOH terminal mini of the kinase, thereby opening up the kinase into an active conformation. Previous studies have also shown that ASK1 is located in the cytoplasm and Daxx is mainly located in the nucleus (13, 15). It is well known that Daxx relocalizes from the nucleus to the cytoplasm in response to stress (15). Thus, relocalization of Daxx is required prior to its interaction with ASK1. A fundamental question is what molecular change(s) regulate the relocalization of Daxx from the nucleus to the cytoplasm in response to stress? Here we provide a possibility that the initial activation of JNK1 during glucose deprivation induces Daxx relocalization through phosphorylation. The relocalized Daxx induces ASK1 oligomerization.

EXPERIMENTAL PROCEDURES

Cell Culture and Glucose Deprivation—Human prostate adenocarcinoma (DU-145) cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen), and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a

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1 The abbreviations used are: TRX, thioredoxin; JNK, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; ASK1, apoptosis signal-regulating kinase 1; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase; GRX, glutaredoxin; HA, hemagglutinin; TNF, tumor necrosis factor; JBD, JNK binding domain; TRAF, TNF-receptor-associated factor; HIPK1, homeodomain-interacting protein kinase 1; PMSF, phenylmethylsulfonyl fluoride; MOI, multiplicity of infection; DTT, dithiothreitol; aa, amino acids; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GST, glutathione S-transferase.
Immune Complex Kinase Assay and in Vivo Labeling—The PCR product of human Daxx containing restriction enzyme sites at the flanking sides (5′, NdeI; 3′, BamHI) was produced using the pFLAG/CMV2-Daxx as a template. Sense primer was 5′-GCTCGATATGCGACCCCATTAACAAGCATAT-3′, and antisense primer was 5′-CTCGGAGTCCCTAATCATGAGGACGAC-3′. pcDNA3.1/His A-Daxx was produced using the PCR product of Daxx with KpnI/EcoRI cut pcDNA3.1-Myc-His A-Daxx. pET15b/Daxx was produced by inserting the SpeI/XbaI fragment from pcDNA3-Myc/ASK1 into SpeI/XbaI-cut pAdlox shuttle vector (16). Various Daxx deletion mutants were FLAG-tagged at their N-terminal and restriction enzyme recognition sites at the flanking sides (5′, EcoRI; 3′, BamHI) produced using the pFLAG/CMV2-Daxx as a template. Sense primer was 5′-GAGCGAATTCACCGCCATTAGACTGACGAC-3′, and antisense primer was 5′-CTCGGAGTCCCTAATCATGAGGACGAC-3′. For Daxx 1–500 (amino acids 1–500), sense primer was the same as that of Daxx 1–625, and antisense primer was 5′-CTATGAGGCTACTCTG-3′. For Daxx 501–625 (amino acids 501–625), sense primer was 5′-GTTGTTAACCTCCCTGCAAAAACTTCGG-3′, and antisense primer was 5′-CTATGAGGCTACTCTGATGAGGACGAC-3′.

Immunoblot Analysis—Cell lysates were subjected to electrophoresis on 10% polyacrylamide gels containing SDS under reducing conditions, and the proteins in the gels were transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with 7% (w/v) skim milk in PBST (PBS containing 0.1% (v/v) Tween 20) and then reacted with primary antibodies. Polyclonal rabbit anti-ACTIVE JNK1 antibody was obtained from Promega (Madison, WI). Monoclonal mouse anti-actin antibody was purchased from ICN. After washing three times with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IGG. Then the proteins were detected with the ECL reagent. The immunoblots were visualized by autoradiography.

Immunofluorescence—Cellular localization of FLAG-Daxx (or His-Daxx) was investigated using fluorescence microscopy. The cells were plated onto a Lab-Tek chamber slide (Nalge Nunc, Naperville, IL) and infected with Ad/FLAG-Daxx (or Ad/His-Daxx) at an MOI of 48 h of infection, cells were fixed in 100% cold methanol for 10 min at −20°C, washed twice with cold PBS, then incubated with 1% bovine serum albumin plus 10% rabbit or goat serum (depending on the source of second antibody) for 1 h at room temperature. They were then incubated with anti-FLAG (clone M2; mouse) or anti-His (penta-His; mouse) antibodies containing 1% bovine serum albumin plus 10% rabbit or goat serum for 1 h at room temperature, followed by three washes with PBS. Samples of Daxx (or His-Daxx) fusion proteins were stained with 1 μg/ml of fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. After washing three times with cold PBS, the slides were mounted in 90% glycerol.
Oligomerization of ASK1 and Interaction between Daxx and ASK1 during Glucose Deprivation—Previous studies have shown that oligomerization of ASK1 occurs during treatment with TNF in human embryonic kidney 293 cells (11). Activation of ASK1 requires reactive oxygen species-mediated dissociation of TRX from ASK1 followed by the binding of TRAF2 and consequent ASK1 homo-oligomerization (11). Thus, we examined whether Daxx also plays a role in the oligomerization of ASK1 during glucose deprivation. Fig. 1A shows that homo-oligomerization of ASK1 occurred during glucose deprivation or \( \text{H}_2\text{O}_2 \) treatment. Unlike ASK1, Daxx existed as an oligomer form irrespective of oxidative stress (Fig. 1B). Several researchers have shown that Daxx, a Fas-binding protein, binds to ASK1, thereby activating the ASK1 kinase (12, 17). We investigated whether glucose deprivation induces interaction between Daxx and ASK1. Fig. 2 shows that Daxx associated with ASK1 during oxidative stress (glucose deprivation or \( \text{H}_2\text{O}_2 \) treatment).

Daxx Binding Site to ASK1 and Localization of Various Deletion Mutant Types of Daxx—We further examined which domain of Daxx is responsible for interacting with ASK1. Cells were co-infected with Ad.HA-ASK1 and Ad.myc-ASK1 at an MOI of 10 (A) or Ad.FLAG-Daxx and Ad.His-Daxx at an MOI of 10 (B). After 48 h of infection, cells were exposed to glucose-free medium for 1 h or \( \text{H}_2\text{O}_2 \) (500 \( \mu \text{M} \)) for 30 min. A, lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-myc or anti-HA antibody (upper panels). The presence of Myc-ASK1 in the lysates was verified by immunoblotting with anti-Myc antibody (lower panel). B, lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-His or anti-FLAG antibody (upper panels). The presence of His-Daxx in the lysates was verified by immunoblotting with anti-His antibody (lower panel). Western blot; IP, immunoprecipitation.

RESULTS

Oligomerization of ASK1 and Interaction between Daxx and ASK1 during Glucose Deprivation—Previous studies have shown that oligomerization of ASK1 occurs during treatment with TNF in human embryonic kidney 293 cells (11). Activation of ASK1 requires reactive oxygen species-mediated dissociation of TRX from ASK1 followed by the binding of TRAF2 and consequent ASK1 homo-oligomerization (11). Thus, we examined whether Daxx also plays a role in the oligomerization of ASK1 during glucose deprivation. Fig. 1A shows that homo-oligomerization of ASK1 occurred during glucose deprivation or \( \text{H}_2\text{O}_2 \) treatment. Unlike ASK1, Daxx existed as an oligomer form irrespective of oxidative stress (Fig. 1B). Several researchers have shown that Daxx, a Fas-binding protein, binds to ASK1, thereby activating the ASK1 kinase (12, 17). We investigated whether glucose deprivation induces interaction between Daxx and ASK1. Fig. 2 shows that Daxx associated with ASK1 during oxidative stress (glucose deprivation or \( \text{H}_2\text{O}_2 \) treatment).

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Interaction between Daxx and ASK1 during oxidative stress. DU-145 cells were co-infected with Ad.HA-ASK1 and Ad.FLAG-Daxx at an MOI of 10, and adenoviral vectors containing FLAG-tagged Daxx (Ad.FLAG-Daxx) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h or 500 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) for 30 min. Lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody (top panels). The presence of FLAG-Daxx in the lysates was verified by immunoblotting with anti-FLAG antibody (bottom panel). Western blot; IP, immunoprecipitation.
shows that oligomerization of ASK1 induced by glucose deprivation was unaffected by overexpressed wild-type Daxx (Fig. 4B, lanes 3 and 5). Unlike wild type Daxx, expression of Daxx deletion mutant (aa 501–625) caused oligomerization of ASK1 even in the presence of glucose (Fig. 4B, lane 6). The differential roles of wild-type Daxx and deletion mutant-type Daxx (aa 501–625) can be explained by differential localization of these proteins (Fig. 3B). Previous studies have shown that Daxx deletion mutant (aa 501–625) is largely responsible for JNK activation (17). Fig. 4C shows that overexpression of Daxx deletion mutant (aa 501–625) indeed activates JNK1 even in the presence of glucose. These results suggest that physical interaction between Daxx and ASK1 is sufficient to cause ASK1 oligomerization and subsequent JNK1 activation.

Effect of JBD Overexpression on Relocalization of Daxx and Interaction between ASK1 and Daxx during Glucose Deprivation—A fundamental question that remains unanswered in this study is how Daxx translocates to the cytoplasm during glucose deprivation. One possibility is that oxidative stress-induced ASK1-SEK1-JNK1 signal transduction is involved in the relocalization of Daxx. We hypothesized that the ASK1-SEK1-JNK1 pathway must already be activated to get increased Daxx binding to ASK1, and then increased Daxx binding would maintain the activated ASK1-SEK1-JNK1. To test this possibility, we overexpressed the JNK binding domain (JBD), a negative regulator of JNK. As shown in Fig. 5, overexpression of JBD inhibited JNK1 activation during glucose deprivation. In contrast, JBD overexpression did not affect the activation of p38 during glucose deprivation (data not shown). Overexpression of JBD prevented relocalization of Daxx to the cytoplasm and the binding of Daxx to ASK1 during glucose deprivation or H2O2 treatment (Fig. 6, A, B, and D). However, Daxx relocalization and interaction between Daxx and ASK1 were not prevented by treatment with SB203580, a specific p38 inhibitor (Fig. 6, C and D). We confirmed the inhibitory effect of SB203580 on p38 by using a p38 MAP kinase assay kit (Cell Signaling Technology, Inc., Beverly, MA) (data not shown). Our results suggest that the oxidative stress-activated ASK1-SEK1-JNK1 signal transduction pathway, but not the ASK1-MKK3/MKK6-p38 pathway, plays an important role in the relocalization of Daxx to the cytoplasm and its subsequent interaction with ASK1.

Glucose Deprivation-induced Daxx Phosphorylation and Effect of JBD Overexpression on Daxx Phosphorylation—To investigate whether Daxx is phosphorylated during glucose deprivation, DU-145 cells were infected with Ad.His-Daxx. Studies from in vivo labeling with [32P]orthophosphate show that Daxx was phosphorylated during glucose deprivation (Fig. 7, lane 3). The phosphorylation of Daxx was suppressed by overexpression of JBD, a negative regulator of JNK (Fig. 7, lane 4). These results suggest that JNK1 is involved in Daxx phosphorylation. Phosphorylation of Daxx in the ASK1-SEK1-JNK1-hipk1 Signal Transduction Pathway Is Mediated during Glucose Deprivation—It is well known that activated JNK can phosphoryl-
shows that activated JNK1 directly phosphorylated c-Jun but not Daxx. These results suggest that activated JNK1 is indirectly involved in Daxx phosphorylation. Recent studies show that homeodomain-interacting protein kinase (HIPK1) physically interacts and directly phosphorylates Daxx (24). We hypothesized that JNK1 activates HIPK1, which then consequently phosphorylates Daxx. An immune complex kinase assay indeed demonstrated that activated JNK1 directly phosphorylated HIPK1 (Fig. 8B) and consequently phosphorylated Daxx (Fig. 8C). These results suggest that glucose deprivation-induced Daxx phosphorylation is mediated through the JNK1-HIPK1 signal transduction pathway.

Role of Serine Residue in the Relocalization of Daxx during Glucose Deprivation—Ecsedy et al. (24) reported that HIPK1 phosphorylates murine Daxx on Ser669, and this amino acid residue plays an important role in the relocalization of Daxx. Unlike murine Daxx, human Daxx contains two serine residues in positions 667 and 670. Based on previous results, we postulated that either serine residue of Daxx plays a role in its relocalization during glucose deprivation. To identify the serine residue that plays an important role in the relocalization of Daxx, we employed site-directed mutagenesis techniques to create one point mutant at two serine residues (Ser → Ala) and evaluated its role in the relocalization of Daxx during glucose deprivation. Fig. 9 shows that S667A mutant type Daxx, but not S670A mutant type Daxx, did not relocalize to the cytoplasm during glucose deprivation.

Effect of JBD Overexpression on ASK1 Oligomerization during Glucose Deprivation—We further investigated the effect of JBD overexpression on ASK1 oligomerization. Fig. 10 shows that glucose deprivation-induced ASK1 oligomerization was inhibited by JBD overexpression (Fig. 10, lane 3 versus lane 5). In contrast, Daxx deletion mutant (aa 501–625)-induced ASK1 oligomerization was not affected by JBD overexpression, regardless of whether glucose was present or absent from the medium. These results suggest that Daxx relocalization is essential for the ASK1 oligomerization during glucose deprivation.

Model for the Role of the ASK1-MAPK-MEK Signal Transduction in Daxx Trafficking during Glucose Deprivation—Fig. 11 shows a schematic diagram of a theoretical model based on the literature and data presented here. According to the model, glucose deprivation elevates the intracellular level of reactive oxygen species, in particular H$_2$O$_2$. Reactive oxygen species activate the ASK1-SEK1-JNK1-HIPK1 signaling pathway, which subsequently signals the relocalization of Daxx from the nucleus to the cytoplasm. The relocalization of Daxx may require its phosphorylation on Ser667 through activated HIPK1. The cytoplasmic Daxx then binds to ASK1 and leads to ASK1 oligomerization.
The goal of our studies was to examine whether ASK-SEK1-JNK1 signaling is responsible for Daxx trafficking. Previous studies have shown that Daxx, which contains two nuclear localization signals, is mainly located in the nucleus of an unstressed cell (13). It interacts with nuclear proteins such as centrometric protein, Pax, and promyelocytic leukemia protein (25). Our data show that Daxx relocalizes from the nucleus to the cytoplasm during glucose deprivation and H$_2$O$_2$ treatment (Figs. 3B and 6D). Overexpression of JBD, which inhibits glucose deprivation-induced JNK1 activation, also suppresses the relocalization of Daxx to the cytoplasm (Figs. 6D). Moreover, overexpression of JBD prevents the glucose deprivation-induced association of Daxx with ASK1 (Fig. 6A). These results suggest that glucose deprivation-activated ASK1-SEK1-JNK1 play an important role in the relocalization of Daxx to the cytoplasm.

**DISCUSSION**

The goal of our studies was to examine whether ASK-SEK1-JNK1 signaling is responsible for Daxx trafficking. Previous studies have shown that Daxx, which contains two nuclear localization signals, is mainly located in the nucleus of an unstressed cell (13). It interacts with nuclear proteins such as centrometric protein, Pax, and promyelocytic leukemia protein (25). Our data show that Daxx relocalizes from the nucleus to the cytoplasm during glucose deprivation and H$_2$O$_2$ treatment (Figs. 3B and 6D). Overexpression of JBD, which inhibits glucose deprivation-induced JNK1 activation, also suppresses the relocalization of Daxx to the cytoplasm (Figs. 6D). Moreover, overexpression of JBD prevents the glucose deprivation-induced association of Daxx with ASK1 (Fig. 6A). These results suggest that glucose deprivation-activated ASK1-SEK1-JNK1 play an important role in the relocalization of Daxx to the cytoplasm.
cytoplasm and its subsequent interaction with ASK1. Recent studies also demonstrate that Daxx requires ASK1 for its cytoplasmic localization (13). Overexpression of ASK1 stimulates the redistribution of Daxx to the cytoplasm (13). The relocalization of Daxx is probably due to an elevated level of JNK1 activity (26). Our data show that Daxx was phosphorylated during glucose deprivation, and its phosphorylation was mediated through HIPK1 activation (Fig. 8). These results are consistent with recent studies that show that HIPK modulates Daxx relocalization and phosphorylation (24). Our data clearly demonstrate that JNK1 activates HIPK1, which then consequently phosphorylates Daxx during glucose deprivation. Our studies also show that Daxx relocalization was inhibited by inhibiting JNK1 activation by JBD overexpression (Figs. 5 and 6). It is possible that Daxx phosphorylation is associated with Daxx export from the nucleus. Recently, several researchers reported that yeast transcription factor, Yap1, a subfamily of AP-1, which is a sensor of the redox state of the cell, is activated by oxidative stress such as H2O2 (27, 28). Activated Yap1 (oxidized form) leads to disulfide bond formation in the C-terminal cysteine-rich region, which contains 3 conserved cysteines and the nuclear export signal. Formation of an intramolecular disulfide linkage leads to a conformational change of Yap1 and consequently conceals the nuclear export signal from the export receptor Crm1p/Xpo1p, resulting in the localization of Yap1p to the nucleus. In contrast to Yap1p in yeast, Daxx in mammalian cells could be exported to the cytoplasm during oxidative stress through an export receptor.
which is similar to Crm1p/Xpo1p in yeast. We postulate that phosphorylation of Daxx results in conformational changes, exposing the nuclear export signal, which would be recognized by an export receptor. Interaction between the nuclear export signal of Daxx and the export receptor may thus be the critical step in redirecting nuclear Daxx to the cytoplasm. Obviously, this model requires substantiation.

It is well known that the C-terminal 112 amino acids of Daxx (aa 626–739) are necessary for Fas binding. However, prior to our study, it was not clear which portion of Daxx is responsible for ASK1 binding. Our data demonstrate that the ASK1 binding site of Daxx resides in the region of amino acids 501–625 (Fig. 3A). Interestingly, we observed that this deletion mutant is mainly localized to the cytoplasm (Fig. 3B). Moreover, overexpression of Daxx 501–625 promotes ASK1 oligomerization as well as JNK1 activation, even in the presence of glucose (Figs. 4C and 10). These results are consistent with previous observations that Daxx 501–625 induces JNK activation as well as apoptotic death (12). These results also suggest that the ASK1 binding site of Daxx plays a role in ASK1 oligomerization and that oligomerization of ASK1 is sufficient for activation of the ASK1-SEK1-JNK1 signal transduction pathway. This observation is supported by data from previous studies, which have shown that TRAF2 enhances ASK1 homo-oligomerization and consequently promotes ASK1 activation (11). Although we are far from understanding how Daxx regulates ASK1 oligomerization, we present the possible role of Daxx in the ASK1-SEK1-JNK1-HIPK1 signal transduction pathway. We hypothesize that activation of the ASK1-SEK1-JNK1-HIPK1 signal promotes relocalization of Daxx, which stabilizes ASK1 oligomerization and maintains the activation of ASK-SEK1-JNK1 signal. If Daxx does not bind to ASK1, activated ASK1 may be quickly inactivated through degradation. We believe that this model will provide a framework for future studies.

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