
Mechanisms of Signal Transduction:

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*J. Biol. Chem. 2003, 278:47245-47252.*
*doi: 10.1074/jbc.M213201200 originally published online September 10, 2003*

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 47, Issue of November 21, pp. 47245–47252, 2003
Printed in U.S.A.


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

Received for publication, December 26, 2002, and in revised form, August 12, 2003
Published, JBC Papers in Press, September 10, 2003, DOI 10.1074/jbc.M213201200

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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 Ser$^{667}$ to Ala results in suppression of Daxx relocalization during glucose deprivation, suggesting that Ser$^{667}$ 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 derivation 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). Dissociation 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-$\alpha$ (10, 11), Fas ligand (12, 13), and endoplasmic reticulum stress (14). Previous studies have shown that TNF-$\alpha$ 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 $NH_2$ and COOH termini 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

* This work was supported by NCI, National Institutes of Health, Grants CA48000, CA95191, and CA96989; the Competitive Medical Research Fund of the University of Pittsburgh Medical Center Health System; the Oral Cancer Center Grant Fund, the Elsa U. Pardee Foundation, the Pittsburgh Foundation; Department of Defense (DOD) prostate program fund Grant PC020530; and a DOD prostate cancer traineeship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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.

humidified atmosphere containing 5% $CO_2$ and air at 37 °C. For glucose deprivation, cells were rinsed three times with phosphate-buffered saline (PBS) and then exposed to glucose-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum (Invitrogen).

*Shuttle Vector Construction*—pFLAG-CMV2-Daxx was kindly provided by Dr. Horikoshi (Mallinckrodt Institute of Radiology in Washington University, St. Louis, MO). pAdlox-FLAG-Daxx was made by inserting the SpeI/BamHI fragment from pFLAG-CMV2-Daxx into SpeI/BamHI-cut pAdlox shuttle vector (16). Various Daxx deletion mutants FLAG-tagged at their N-terminal and restriction enzyme recognition sites at the flanking sides (5′, EcoRI; 3′, BamHI) were produced by PCR. For Daxx 1–625 (amino acids 1–625), sense primer was 5′-GAGCGAATTCAGCCACCGCTAACAGCATCATC-3′ and antisense primer was 5′-CTGCGGATCCCTAACCAGAATCTCCCCAGTTGTG-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′-CTATGGATCCCTACTGTAGTGAGGACATGGGGCTC-3′. For Daxx 501–625 (amino acids 501–625), sense primer was 5′-GCTCGAATTCAATCTCCAATG-AAAAGAACCTGG-3′, and antisense primer was the same as that of Daxx 1–625. For Daxx 626–739 (amino acids 626–739), sense primer was 5′-GGTTGAATTCACCCCCCTGCAAAAATCTCGG-3′, and antisense primer was 5′-CTATGGATCCCTAATCAGAGTCTGAGAGCAC-3′. pcDNA3.1/His A-Daxx was made by inserting the PCR product of Daxx digested with KpnI/EcoRI to KpnI/EcoRI-cut pcDNA3.1/His A. Sense primer was CTGTGGTACCTGCCACCGCTAACAGCATCATC-3′, and antisense primer was 5′-GACTGAATTCCTAATCAGAGTCTGAGAGCACG-3′. pAdlox-His-Daxx was made by inserting SpeI/EcoRI fragment from pcDNA3.1-His A-Daxx into SpeI/EcoRI-cut pAdlox. pcDNA3-HA-ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox-HA-ASK1 was made by inserting the SpeI/XbaI fragment from pcDNA3-HA-ASK1 into XbaI-cut pAdlox. pcDNA3-Myc-ASK1 was made by inserting the PCR product of ASK1 to pcDNA3-Myc. Sense primer was 5′-ATTATACGTATAGCACGGAGGCGGACGAGGG-3′ introducing a SnaBI site, and antisense primer was 5′-CGCGTCTAGATCAAGTCTGTTTGTTTCGAAAGTCAATG-3′ introducing a XbaI site for inserting into pcDNA3-Myc (BamHI → Klenow → XbaI). pAdlox-Myc-ASK1 was made by inserting the SpeI/XbaI fragment from pcDNA3-Myc-ASK1 into SpeI/XbaI-cut pAdlox. pAdlox-FLAG-JBD was made by inserting the SpeI/XbaI fragment from pcDNA3-FLAG-JBD into SpeI/XbaI-cut pAdlox. pAdlox-His-JNK1 was also made by inserting the SpeI/XbaI fragment from pcDNA3.1-His A-JNK1 into SpeI/XbaI-cut pAdlox.

*Adenoviral Vector Construction*—All recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system (16). The selective cell line CRE8 has a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using LipofectAMINE Reagent (Invitrogen). $5 \times 10^5$ cells were split into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μg of SfiI/ApaI-digested Adlox/HA or Myc-ASK1 fragment or Adlox/FLAG-JBD or SfiI-digested Adlox/FLAG-Daxx including various Daxx deletion mutants or His-Daxx fragment or Adlox/His-JNK1 and 2 μg of ψ5 viral genomic DNA were co-transfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ5 viral DNA. A new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of HA-ASK1 or Myc-ASK1 or FLAG-JBD or various FLAG-Daxx or His-Daxx or His-JNK1 to adenovirus was confirmed by Western blot analysis, after infection of corresponding recombinant adenovirus into DU-145 cells.

*In Vivo Binding of ASK1 with Daxx*—To examine the interaction between ASK1 and Daxx, adenovirus of HA-tagged ASK1 (Ad.HA-ASK1) and FLAG-tagged Daxx (Ad.FLAG-Daxx) at an MOI of 10 were co-infected into DU-145 cells in 10-cm culture plates. For immunoprecipitation, after 48 h of infection, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 80 μM aprotinin, 2 mM leupeptin, and the lysates were incubated with 3 μg of anti-FLAG M2 mouse IgG1 (Sigma) or 0.5 μg of rat anti-HA (clone 3F10; Roche Applied Science) for 2 h, respectively. After the addition of protein G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS-PAGE, and immunoblotted with rat anti-HA or mouse anti-HA (clone 12CA5; Roche Applied Science) antibodies or mouse anti-FLAG. Proteins in the membranes were then visualized using the ECL reagent as recommended by the manufacturer (Amersham Biosciences).

*Immune Complex Kinase Assay and in Vivo Labeling*—The PCR product of human Daxx having 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′-GCTGCATATGGCCACCGCTAACAGCATCATC-3′, and antisense primer was 5′-CTGCGGATCCCTAATCAGAGTCTGAGAGCAC-3′. pET15b/Daxx was made by inserting NdeI/BamHI fragment from Daxx PCR product into NdeI/BamHI-cut pET15b (Novagen, Madison, WI). pET15b/Daxx was transformed into BL21(DE3)pLysS, and Daxx expression was confirmed by anti-Daxx (Sigma). His-Daxx was purified by using His-bind column (Novagen). For the immune complex kinase assay, DU-145 cells were infected with Ad.His-JNK1 at an MOI of 10. After 48 h of infection, cells were in glucose-free medium for 1 h and lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse anti-His antibody (Qiagen, Valencia, CA) and protein G-agarose. The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution and washed once with the kinase buffer solution, and then they were subjected to kinase assays. To examine whether the Daxx was a direct substrate of JNK1, 1 μg of His-Daxx or GST-c-Jun was incubated with immunoprecipitated His-JNK1 in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT, 20 μM ATP, and 100 μCi/ml [$\gamma$-$^{32}$P]ATP at 30 °C for 1 h. Finally, the reaction was stopped by adding 2× Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography. For direct [$^{32}$P]orthophosphate labeling of His-Daxx, DU-145 cells were infected with Ad.His-Daxx at an MOI of 10. After 48 h of infection, cells were pre-equilibrated in phosphate-free medium for 3 h prior to the addition of 50 μCi/ml of [$^{32}$P]orthophosphate (ICN, Irvine, CA) in phosphate and glucose-free medium for 1 h and then lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse anti-His antibody and protein G-agarose. The beads were washed three times with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution. The immune complex was separated by SDS-PAGE, and dried gel was visualized by autoradiography.

*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 polyvinylidine 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 JNK 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.

*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 10. After 48 h of infection, cells were fixed in 100% cold methanol for 10 min at −20 °C. After washing twice with cold PBS, the cells were blocked in 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 cold PBS. Samples of FLAG-Daxx (or His-Daxx) were incubated for 1 h with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG. After washing three times with cold PBS, the slides were mounted in 90% glycerol.

*Production of HIPK1 Protein*—To prepare HIPK1 substrate proteins for kinase assay, a plasmid containing GST-HIPK1 was constructed in the EcoRI/SmaI site of pGEX-4T-2 by inserting the BamHI/EcoRI fragment from pcDNA3.1Myc.His-HIPK1 (kindly provided by P. Leder (Howard Hughes Medical Institute, Boston, MA). pGEX-4T-2/HIPK1 was transformed into JM109, and HIPK1 expression was confirmed by anti-HIPK1 antibody (kindly provided by P. Leder) and purified by

using glutathione-Sepharose 4B (Amersham Biosciences).

*Site-directed Mutagenesis*—The QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make point mutations in Daxx protein. Two serine residues in Daxx ($Ser^{667}$ and $Ser^{670}$) were replaced with alanine. Sense primer oligonucleotide (5′-GTACCCTGCCCGCCCCACCTTCCC-3′) and antisense primer oligonucleotide (5′-GGGAAGGTGGGGCGGGCAGGGTAC-3′) were for S667A. Sense primer oligonucleotide (5′-GCCCAGCCCACCTGCCCCCTTAGCTTC-3′) and antisense primer oligonucleotide (5′-GAAGCTAAGGGGGCAGGTGGGCTGGGC-3′) were for S670A. PCR was prepared by adding 5 μl of 10× reaction buffer, 20 ng of double-stranded DNA template (pFLAGCMV2-Daxx), 125 ng of each sense primer, 125 ng of each antisense primer, 1 μl of dNTP mix, double-distilled water to a final volume of 50 μl, and 1 μl of *Pfu* Turbo DNA polymerase (2.5 units/μl). PCR was performed with 12 cycles (95 °C for 30 s; 55 °C for 1 min; 68 °C for 14 min for S667A and 95 °C for 30 s; 58 °C for 1 min; 68 °C for 14 min for S670A) with an initial incubation at 95 °C for 30 s. Following temperature cycling, the reaction was placed on ice for 2 min to cool the reaction. After PCR, 1 μl of DpnI restriction enzyme (10 units/μl) was added directly to each amplification reaction and incubated at 37 °C for 1 h to digest the parental supercoiled double-stranded DNA. The DpnI-treated double-stranded DNA was transformed into *Epicurian Coli* XL1-Blue supercompetent cells. Colonies were selected, and each plasmid (pFLAGCMV2-Daxx) was subcloned into pAdlox-Flag-Daxx, followed by digestion with HindIII/KpnI. Its fragment containing the mutation site was subcloned into pBluescript SK(−). Each pBluescript SK(−)-Daxx fragment was sequenced using T7 primer to confirm mutation.

## 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. 1*A* shows that homo-oligomerization of ASK1 occurred during glucose deprivation or $H_2O_2$ treatment. Unlike ASK1, Daxx existed as an oligomer form irrespective of oxidative stress (Fig. 1*B*). 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 $H_2O_2$ 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 adenoviral vectors containing wild-type Daxx or its various Daxx deletion mutants (amino acids 1–625, 1–500, 501–625, and 626–739). Fig. 3*A* shows that wild-type Daxx and Daxx deletion mutants (aa 1–625 and 501–625) interacted with ASK1 during glucose deprivation. Fig. 3*B* shows that wild-type Daxx, which contains two nuclear localization signals (18), is mainly located in the nucleus. Wild-type Daxx relocalized to the cytoplasm during glucose deprivation. In contrast, the Daxx deletion mutant (aa 501–625) is mainly located in the cytoplasm irrespective of the extracellular glucose concentration. Other deletion mutants (aa 1–625, 1–500, and 626–739), which contain one nuclear localization signal, localize to both the cytoplasm and the nucleus (Fig. 3*B*). Glucose deprivation did not significantly alter the intracellular distribution of any of the deletion mutants (Fig. 3*B*). These results suggest that intracellular location of Daxx as well as amino acid residues of the Daxx domain play an important role in the interaction between Daxx and ASK1. This possibility was further examined. As shown previously in Fig. 2, wild-type Daxx associated with ASK1 during glucose deprivation (Fig. 4*A*, *lane 3*). However, unlike wild-type Daxx, Daxx deletion mutant (aa 501–625) interacted with ASK1 regardless of whether glucose was present or absent from the medium (Fig. 4*A*, *lanes 4* and *5*). We also investigated whether Daxx is involved in the homo-oligomerization of ASK1. Fig. 4*B*

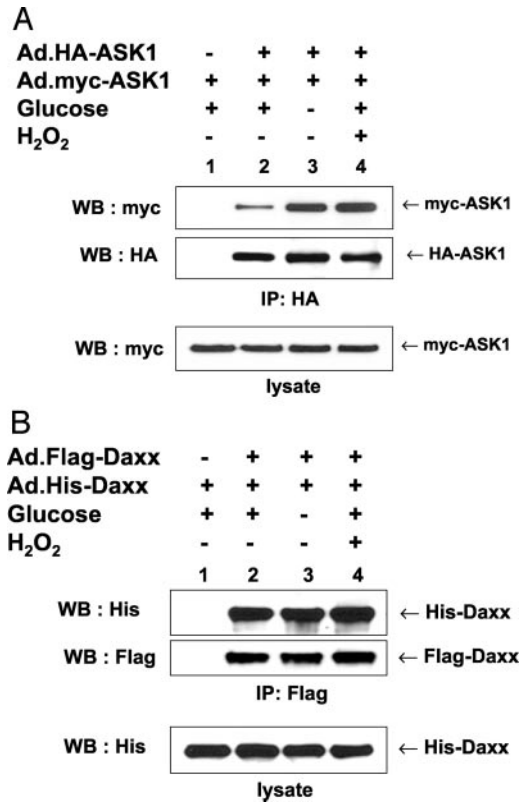


FIG. 1. **Oligomerization of ASK (*A*) or Daxx (*B*) during glucose deprivation or $H_2O_2$ treatment in DU-145 cells.** Cells were co-infected with Ad.HA-ASK1 and Ad.Myc-ASK1 at an MOI of 10 (*A*) or Ad.FLAG-Daxx and Ad.His-Daxx at an MOI of 10 (*B*). After 48 h of infection, cells were exposed to glucose-free medium for 1 h or $H_2O_2$ (500 μM) 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*). *WB*, Western blot; *IP*, immunoprecipitation.

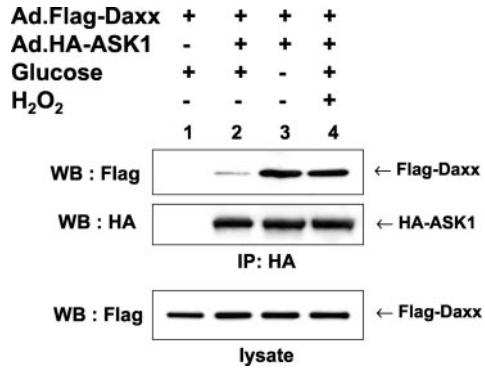


FIG. 2. **Interaction between Daxx and ASK1 during oxidative stress.** DU-145 cells were co-infected with Ad.HA-ASK1 at an MOI of 10, and adenoviral vectors containing FLAG-tagged Daxx (Ad.FLAG-Daxx) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h or 500 μM of $H_2O_2$ 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*). *WB*, Western blot; *IP*, immunoprecipitation.

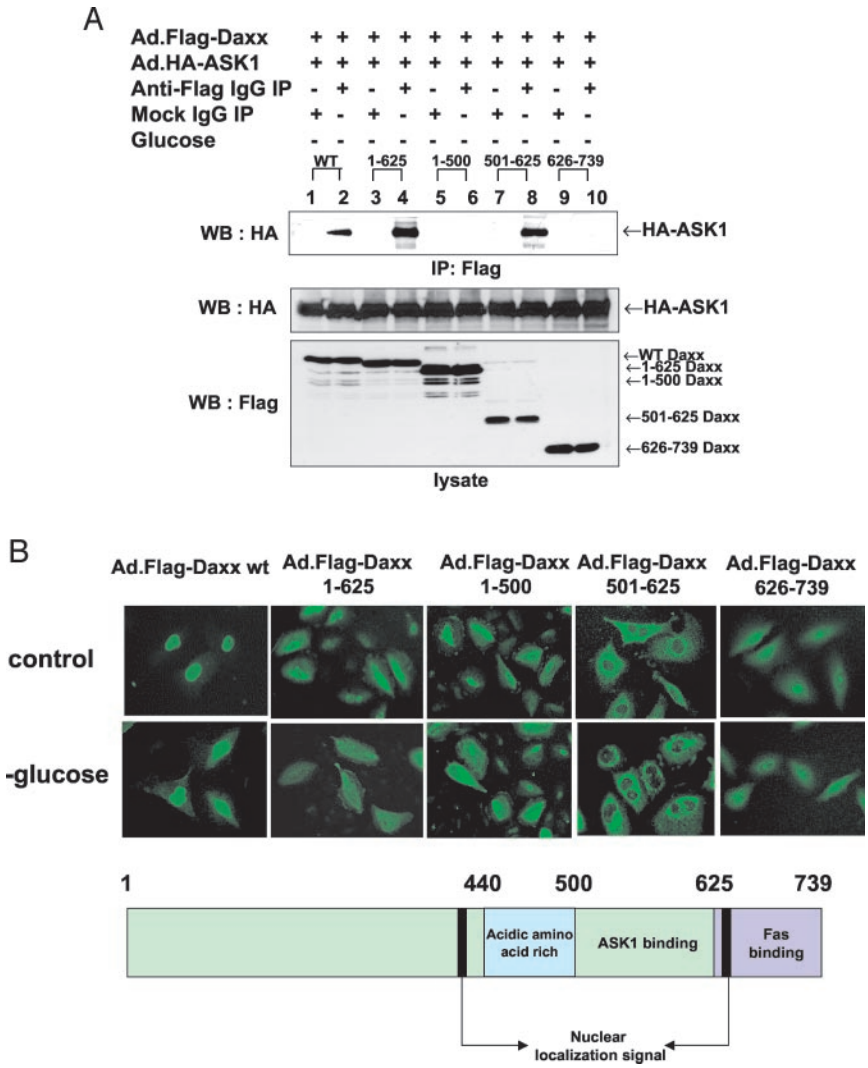


FIG. 3. **Effect of deletion mutant-type Daxx on interaction between Daxx and ASK1 and localization of Daxx.** *A*, cells were co-infected with Ad.HA-ASK1 and adenoviral vectors containing FLAG-tagged wild-type Daxx (*WT*) or various deletion mutant types of Daxx (amino acids 1–625, 1–500, 501–625, or 626–739) at an MOI of 10. After 48 h of infection, cells were exposed to complete medium or glucose-free medium for 1 h. Lysates were immunoprecipitated with anti-FLAG or mock antibody and immunoblotted with anti-HA antibody (*upper panel*). The presence of HA-ASK1 or FLAG-Daxx in the lysates was verified by immunoblotting with anti-HA or anti-FLAG antibody (*lower panels*). *B*, cells were infected with Ad.FLAG-Daxx (wild-type or various deletion mutant-types of Daxx) at an MOI of 10. After 48 h of infection, cells were exposed to complete medium or glucose-free medium for 1 h. The subcellular localization of Daxx was determined by immunofluorescent staining with anti-FLAG antibody (*upper panels*). A schematic diagram of Daxx is shown (*lower panel*). *WB*, Western blot; *IP*, immunoprecipitation.

shows that oligomerization of ASK1 induced by glucose deprivation was unaffected by overexpressed wild-type Daxx (Fig. 4*B*, *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. 4*B*, *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. 3*B*). Previous studies have shown that Daxx deletion mutant (aa 501–625) is largely responsible for JNK activation (17). Fig. 4*C* 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 $H_2O_2$ 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 [$^{32}$P]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-

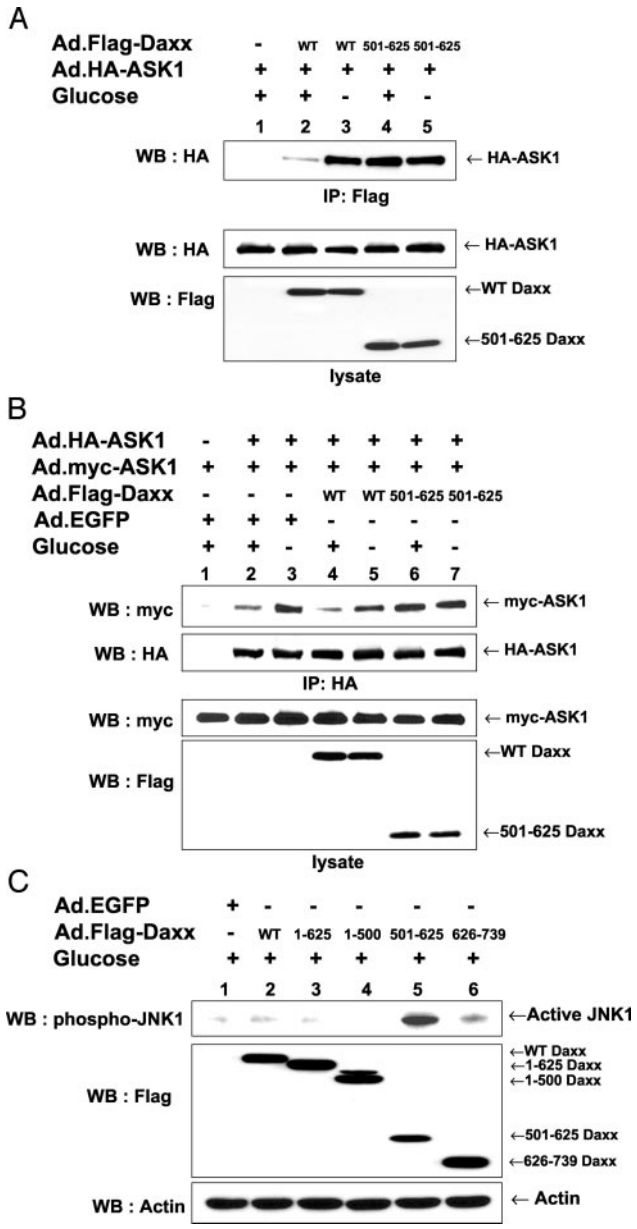


FIG. 4. **Effect of 501–625 deletion mutant-type Daxx on interaction between Daxx and ASK1, oligomerization of ASK1, and JNK1 activation in DU-145 cells.** *A*, cells were co-infected with Ad.HA-ASK1 and adenoviral vectors containing FLAG-tagged wild-type Daxx (WT) or 501–625 deletion mutant-type Daxx at an MOI of 10. After 48 h of infection, cells were exposed to complete medium or glucose-free medium for 1 h. Lysates were immunoprecipitated with anti-FLAG, and immunoblotted with anti-HA antibody (*upper panel*). The presence of HA-ASK1 or FLAG-Daxx in the lysates was verified by immunoblotting with anti-HA or anti-FLAG antibody (*lower panels*). *B*, cells were co-infected with Ad.HA-ASK1 and Ad.Myc-ASK1 at an MOI of 10 and then Ad.FLAG-Daxx (either wild-type Daxx or deletion mutant-type Daxx (aa 501–625) or Ad.EGFP at an MOI of 10. After 48 h of infection, cells were exposed to complete medium or glucose-free medium for 1 h. Lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc or anti-HA antibody (*upper panels*). The presence of Myc-ASK1 or FLAG-Daxx in the lysates was verified by immunoblotting with anti-Myc or anti-FLAG antibody (*lower panels*). *C*, cells were infected with either Ad.EGFP or Ad.FLAG-Daxx (wild-type or various mutant types of Daxx) at an MOI of 10. After 48 h of infection, cell lysates were immunoblotted with anti-active JNK, anti-FLAG, or anti-actin antibody. Actin is shown as an internal standard. *WB*, Western blot; *IP*, immunoprecipitation.

ate various kinds of transcription factors such as ATF2 (19), c-Jun (20), Elk1 (21), Sap1 (22), and p53 (23). Thus, we examined whether JNK1 directly phosphorylates Daxx. Fig. 8*A*

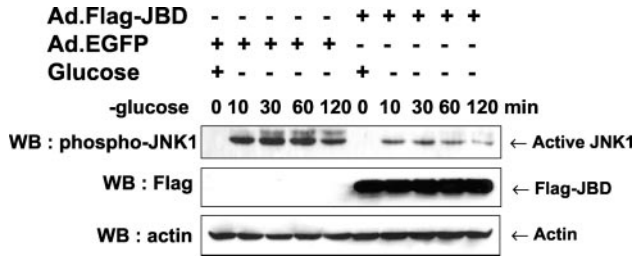


FIG. 5. **Effect of JBD overexpression on glucose deprivation-induced JNK1 activation.** Cells were infected with either adenoviral vectors containing FLAG-tagged JBD (Ad.FLAG-JBD) or Ad.EGFP at an MOI of 100. After 48 h of infection, cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates containing equal amounts of protein (20 $\mu$g) were separated by SDS-PAGE and immunoblotted with anti-active JNK antibody, anti-FLAG antibody, or anti-actin antibody. Actin was used to confirm the amount of protein loaded in each lane. *WB*, Western blot.

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. 8*B*) and consequently phosphorylated Daxx (Fig. 8*C*). 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 $Ser^{669}$, 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 $H_2O_2$. 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 $Ser^{667}$ through activated HIPK1. The cytoplasmic Daxx then binds to ASK1 and leads to ASK1 oligomerization.

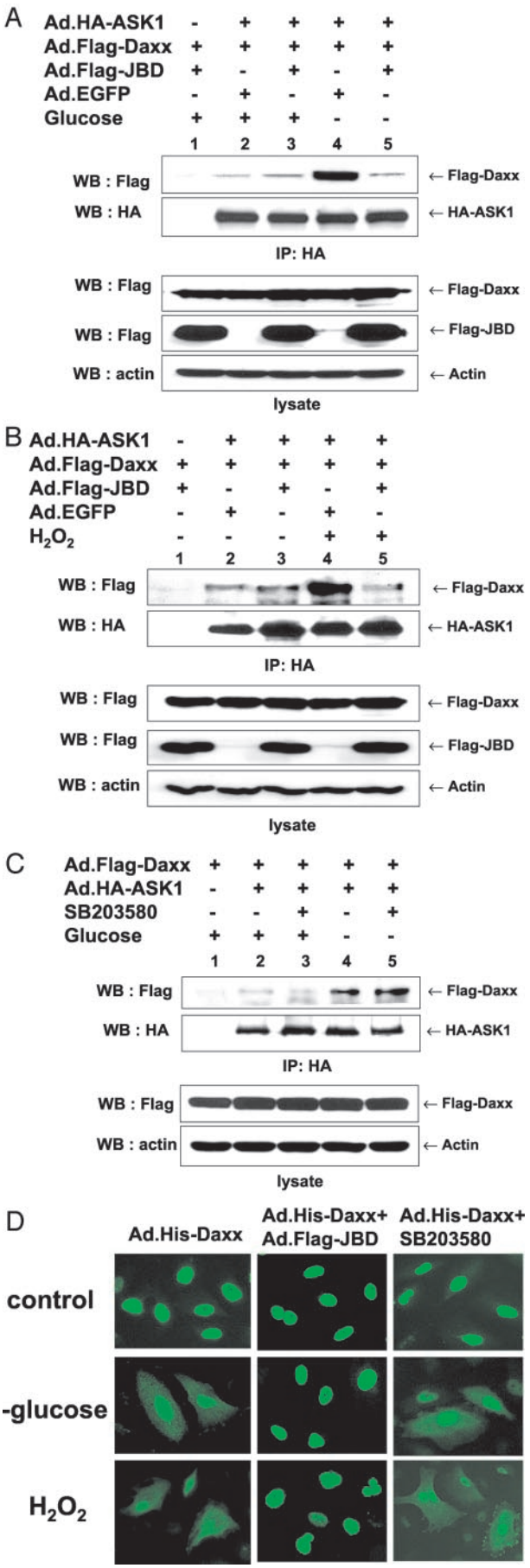


FIG. 6. Effect of JBD overexpression (*A* and *B*) or SB203580 (*C*) on the interaction between ASK1 and Daxx in DU-145 cells. *A* and *B*, cells were infected with Ad.HA-ASK1 at an MOI of 10, Ad.FLAG-Daxx at an MOI of 10, and Ad.FLAG-JBD/Ad.EGFP at an MOI of 100. After 48 h of incubation, cells were exposed to glucose-free medium for 1 h (*A*) or $H_2O_2$ (500 $\mu$M) (*B*) for 30 min. Lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody (*upper panels*). The presence of FLAG-Daxx, FLAG-JBD, or actin in the lysates was verified by immunoblotting with anti-FLAG or anti-actin antibody (*lower panels*). *C*, cells were co-infected with Ad.FLAG-Daxx at an MOI of 10 and Ad.HA-ASK1 at an MOI of 10. After 48 h of infection, cells were treated with SB203580 (10 $\mu$M) for 30 min before and during glucose deprivation for 1 h. Lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody (*upper panels*). The presence of FLAG-Daxx or actin in the lysates was verified by immunoblotting with anti-FLAG or anti-actin antibody (*lower panels*). Actin is shown as an internal standard. *D*, cells were infected with Ad.His-Daxx alone (*left panels*), co-infected with Ad.His-Daxx at an MOI of 10 and Ad.FLAG-JBD at an MOI of 100 (*middle panels*), or infected with Ad.His-Daxx at an MOI of 10 and treated with SB203580 (10 $\mu$M) 30 min before and during glucose deprivation or $H_2O_2$ treatment (*right panels*). After 48 h of infection, cells were exposed to either glucose-free medium for 1 h or $H_2O_2$ (500 $\mu$M) for 30 min. The subcellular localization of Daxx was determined by immunofluorescent staining with anti-His antibody. *WB*, Western blot; *IP*, immunoprecipitation.

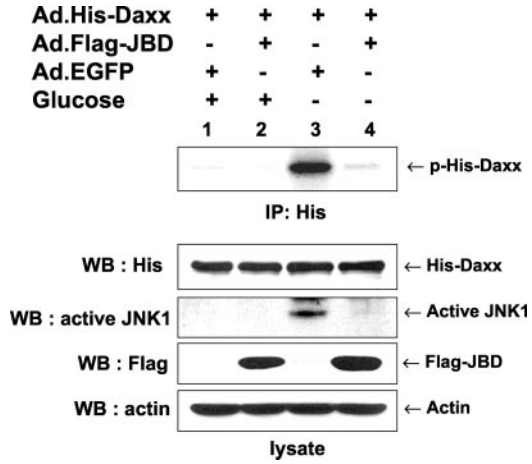


FIG. 7. **Effect of JBD overexpression on glucose deprivation-induced Daxx phosphorylation.** Du-145 cells were infected with Ad.His-Daxx at an MOI of 10 and Ad.FLAG-JBD/Ad.EGFP at an MOI of 100. After 48 h of infection, cells were pre-equilibrated in phosphate-free medium for 3 h prior to the addition of 50 $\mu$Ci/ml [$^{32}$P]orthophosphate in phosphate and glucose-free medium for 1 h and lysed. Cell lysates were divided into two portions. One portion was immunoprecipitated with anti-His antibody, and the immune complex was separated by SDS-PAGE. The dried gel was visualized by autoradiography (*upper panel*). The other portion was immunoblotted with anti-His, anti-active-JNK, anti-FLAG, or anti-actin antibody (*lower panels*). Actin was used to confirm that similar amounts of proteins were loaded in each lane. WB, Western blot; IP, immunoprecipitation.

## 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 $H_2O_2$ treatment (Figs. 3*B* and 6*D*). Overexpression of JBD, which inhibits glucose deprivation-induced JNK1 activation, also suppresses the relocalization of Daxx to the cytoplasm (Figs. 6*D*). Moreover, overexpression of JBD prevents the glucose deprivation-induced association of Daxx with ASK1 (Fig. 6*A*). These results suggest that glucose deprivation-activated ASK1-SEK1-JNK1 play an important role in the relocalization of Daxx to the

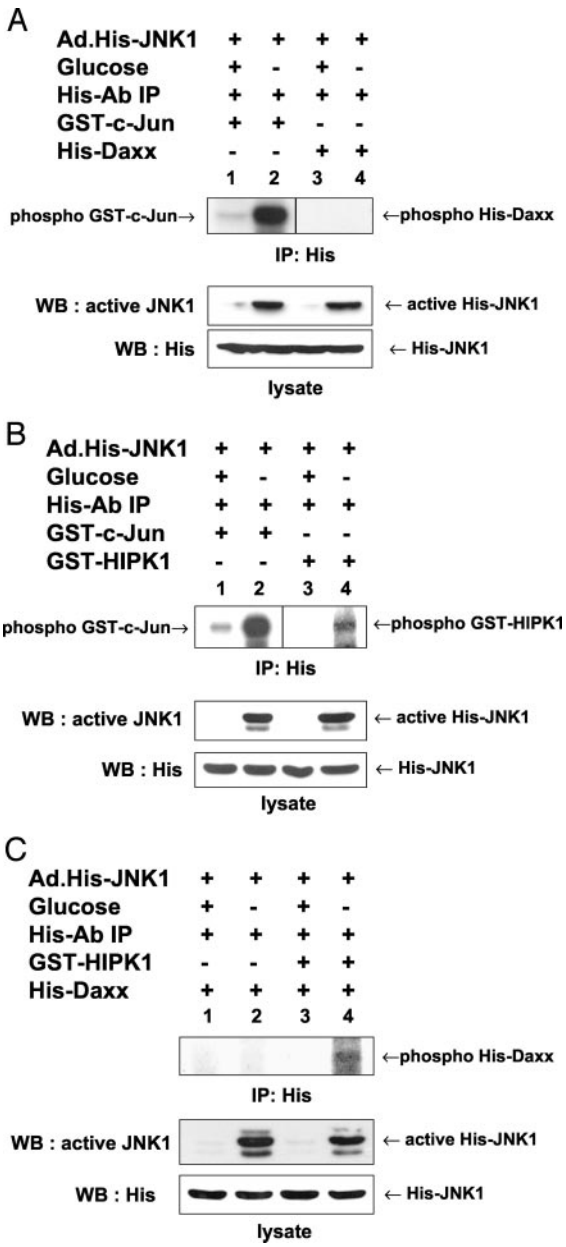


FIG. 8. **Phosphorylation of Daxx is mediated by JNK1-HIPK1 during glucose deprivation.** DU-145 cells were infected with Ad.His-JNK1 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h and lysed. Cell lysates were divided into two portions. One portion was immunoprecipitated with mouse anti-His antibody. *A*, to examine whether the Daxx is a direct substrate of JNK1, 1 $\mu$g of His-Daxx or GST-c-Jun was incubated with immunoprecipitated His-JNK1 in kinase buffer containing 100 $\mu$Ci/ml [$\gamma$-$^{32}$P]ATP at 30 °C for 1 h. *B*, to examine whether the HIPK1 is a direct substrate of JNK1, 0.5 $\mu$g of GST-HIPK1 or 1 $\mu$g of GST-c-Jun was incubated with immunoprecipitated His-JNK1 in kinase buffer containing 100 $\mu$Ci/ml [$\gamma$-$^{32}$P]ATP at 30 °C for 1 h. *C*, to examine whether the Daxx is a sequential substrate of HIPK1, 0.1 $\mu$g of GST-HIPK1 was incubated with immunoprecipitated His-JNK1 in kinase buffer containing 100 $\mu$M ATP at 30 °C for 30 min and subsequently with 1 $\mu$g of His-Daxx with 100 $\mu$Ci/ml [$\gamma$-$^{32}$P]ATP at 30 °C for an additional 30 min. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography (*upper panel*). The other portion was immunoblotted with anti-active JNK or anti-His antibody (*lower panels*). *WB*, Western blot; *IP*, immunoprecipitation.

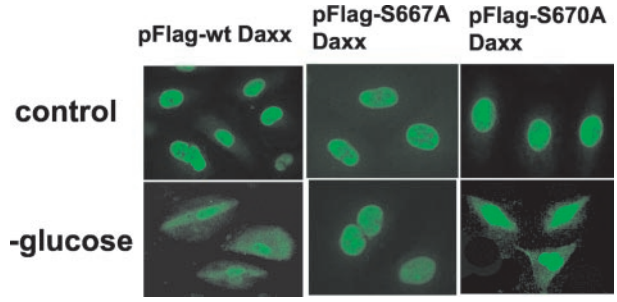


FIG. 9. **Role of serine 667 of Daxx in glucose deprivation-induced Daxx relocalization.** DU145 cells were transfected with pFLAGCMV2-Daxx (wild type), pFLAGCMV2-Daxx (S667A), or pFLAGCMV2-Daxx (S670A). After 48 h of transfection, cells were exposed to glucose-free medium for 1 h. The subcellular localization of Daxx was determined by immunofluorescent staining with anti-FLAG antibody.

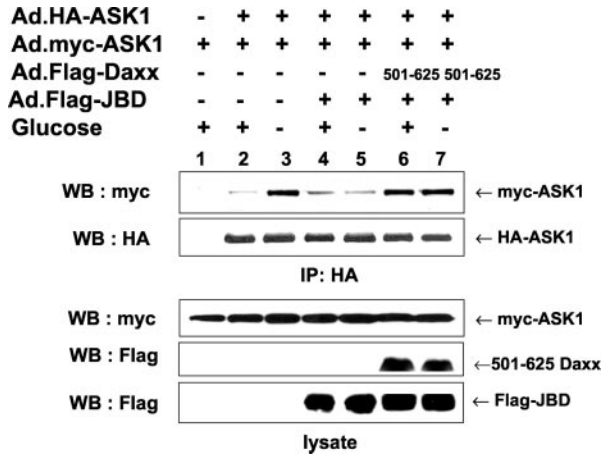


FIG. 10. **Effect of JBD overexpression on the oligomerization of ASK1 during glucose deprivation in DU-145 cells.** Cells were co-infected with Ad.HA-ASK1, Ad.Myc-ASK1, and Ad.FLAG-Daxx at an MOI of 10 and then Ad.FLAG-JBD at an MOI of 100. After 48 h of infection, cells were exposed to complete medium or glucose-free medium for 1 h. Lysates were immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted with anti-Myc or anti-HA antibody (*upper panels*). The presence of Myc-ASK1, FLAG-Daxx (aa 501–625), or FLAG-JBD in the lysates was verified by immunoblotting with anti-Myc or anti-FLAG antibody (*lower panels*). *WB*, Western blot.

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 HPIK modulates Daxx relocalization and phosophorylation (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*D*). 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 $H_2O_2$ (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,

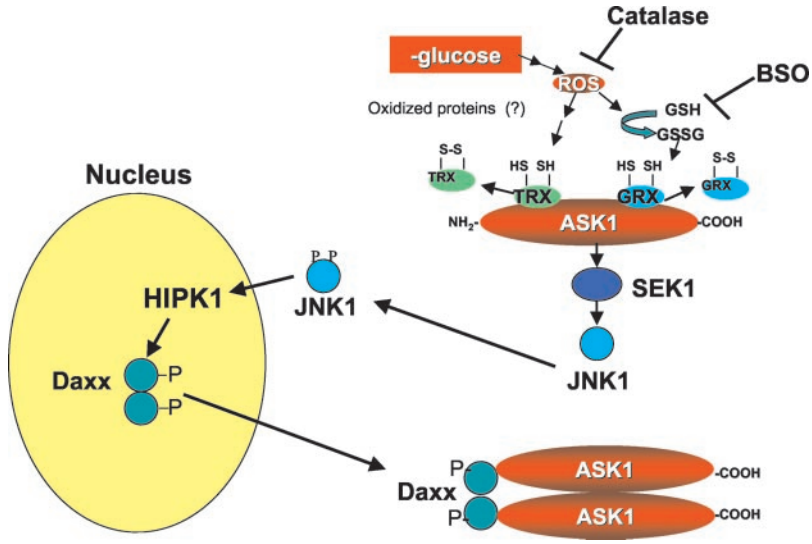


FIG. 11. **A theoretical model for the mechanism by which Daxx is involved in glucose deprivation-induced ASK1-SEK1-JNK1-HIPK1 signal transduction.** This model illustrates that Daxx is phosphorylated and relocalized from the nucleus to the cytosol through the ASK1-SEK1-JNK1-HIPK1 signal transduction pathway during glucose deprivation. Daxx binds to ASK1 and may stabilize the form of ASK1 oligomer.

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. 3*A*). Interestingly, we observed that this deletion mutant is mainly localized to the cytoplasm (Fig. 3*B*). Moreover, overexpression of Daxx 501–625 promotes ASK1 oligomerization as well as JNK1 activation, even in the presence of glucose (Figs. 4*C* 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 oliogomerization 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.

*Acknowledgments*—We thank Patricia M. Rice for skillful technical assistance. We are also grateful to Sandra S. Galoforo and Dr. Charles Vidair for critical reading of the manuscript and stimulating discussion.

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