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Glucose Deprivation**

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## Tryptophan 621 and Serine 667 Residues of Daxx Regulate Its Nuclear Export during Glucose Deprivation\*

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**The cellular target responsible for the nuclear export of Daxx has been identified as chromosomal region maintenance 1 (CRM1), which is a carrier protein for nuclear export and a receptor for the nuclear export signal (NES) of Daxx. Binding of Daxx to CRM1 was increased early during glucose deprivation and then gradually decreased. This interaction was inhibited by leptomycin B, a specific inhibitor of CRM1-dependent nuclear export. Substitution of the serine 667 amino acid residue of Daxx with alanine reduced the interaction with CRM1 during glucose deprivation, suggesting that the phosphorylation of Ser-667 is required for its binding to CRM1 and for its subsequent nuclear export. Data from coupled transcription-translation studies reveal that the NES (amino acids 565–575) of Daxx is a binding site for CRM1. Interestingly, constitutive export of Daxx has occurred by replacement of the tryptophan 621 Daxx residue with alanine. These results suggest that this tryptophan residue plays a key role in masking the NES of Daxx from its receptor, CRM1, in the resting state, whereas phosphorylation of serine 667 would release the NES, which could then be recognized by the CRM1.**

Daxx is a nuclear protein that normally interacts with promyelocytic leukemia protein and localizes to promyelocytic leukemia oncogenic domains (1, 2). Under certain circumstances, Daxx associates with cytoplasmic and cell surface molecules, including transforming growth factor- $\beta$  and Fas (3, 4). Daxx binds to the Fas death domain and induces apoptotic signaling by interacting with ASK1<sup>1</sup> (4, 5). Recently we reported that phosphorylation of Daxx occurs during glucose deprivation, and its phosphorylation is mediated through the ASK1-SEK1-JNK1-HIPK1 signal transduction pathway (6). Phosphorylated

Daxx is translocated from the nucleus to the cytoplasm, binds to ASK1, and subsequently leads to ASK1 oligomerization (6). Phosphorylation at serine 667 of Daxx is required for relocalization of Daxx during glucose deprivation (6). Although Daxx translocation is associated with the phosphorylation of its specific serine residue, it still remains obscure how the export of Daxx is regulated during glucose deprivation.

Several researchers reported that chromosomal region maintenance 1 (CRM1) (also known as exportin 1) is an evolutionarily conserved nuclear export factor (7–11) and acts as a receptor for the nuclear export signal (NES) in both lower and higher eukaryotes. In fact, Daxx contains a putative consensus sequence with a typical leucine-rich (in bold) NES for a functional Rev/Rex NES (amino acids 565–575 **LFELEIEALPL**) (12). In the present study, we hypothesized that CRM1 binds to phosphorylated Daxx and leads to translocation of Daxx. To test this possibility, we investigated the role of phosphorylation at Ser-667 of Daxx in the interaction between Daxx and CRM1 during glucose deprivation. We provide evidence that phosphorylation at Ser-667 of Daxx promotes the association of Daxx with CRM1 and its translocation. In addition, constitutive relocation of Daxx and its association with CRM1 were observed when Trp-621 was replaced with Ala. Taken together, we propose a model for the mechanism of the dual regulation of Daxx-CRM1 interaction by Ser-667 and Trp-621. Trp-621 plays a key role of preventing the interaction between Daxx and CRM1, whereas phosphorylation of Ser-667 promotes the interaction between them.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—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 humidified atmosphere containing 5% CO<sub>2</sub> and air at 37 °C.

**Reagents and Antibodies**—Monoclonal antibodies were purchased from the following companies: anti-HA (clone 3F10) from Roche Diagnostics, anti-FLAG from Sigma, and anti-CRM1 from BD Biosciences. Other chemicals were purchased from Sigma.

**Site-directed Mutagenesis**—The QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in Daxx protein. One tryptophan residue in Daxx (Trp-621) was replaced with alanine (W621A). A sense primer oligonucleotide (5'-CTCTCCTCACACGCG-GGAGATTCTGGTCC-3') and antisense primer oligonucleotide (5'-GG-ACCAGAATCTCCCGCGTTGTGAGGAGAG-3') were used for site-directed mutagenesis. The PCR reaction mixture was prepared by adding 5  $\mu$ l of 10 $\times$  reaction buffer, 20 ng of double-stranded DNA template (pAdlox-FlagDaxx), 125 ng of each sense primer, 125 ng of each antisense primer, 1  $\mu$ l of dNTP mix, double-distilled water to a final volume of 50  $\mu$ l, and 1  $\mu$ l of *Pfu* Turbo DNA polymerase (2.5 units/ $\mu$ l). PCR was performed for 16 cycles (95 °C for 30 s; 55 °C for 1 min; 68 °C for 14 min) with the 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  $\mu$ l of DpnI restriction enzyme (10 units/ $\mu$ l) 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®

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<sup>1</sup> The abbreviations used are: ASK1, apoptosis signal-regulating kinase 1; SEK1, stress-activated protein kinase/extracellular-signal regulated kinase kinase; JNK1, c-Jun N-terminal kinase 1; CRM1, chromosomal region maintenance 1; HIPK1, homeodomain-interacting protein kinase 1; PBS, phosphate-buffered saline; HA, hemagglutinin; NES, nuclear export signal; m.o.i., multiplicity of infection; BSA, bovine serum albumin; Yap1p, yeast AP-1-like transcription factor.

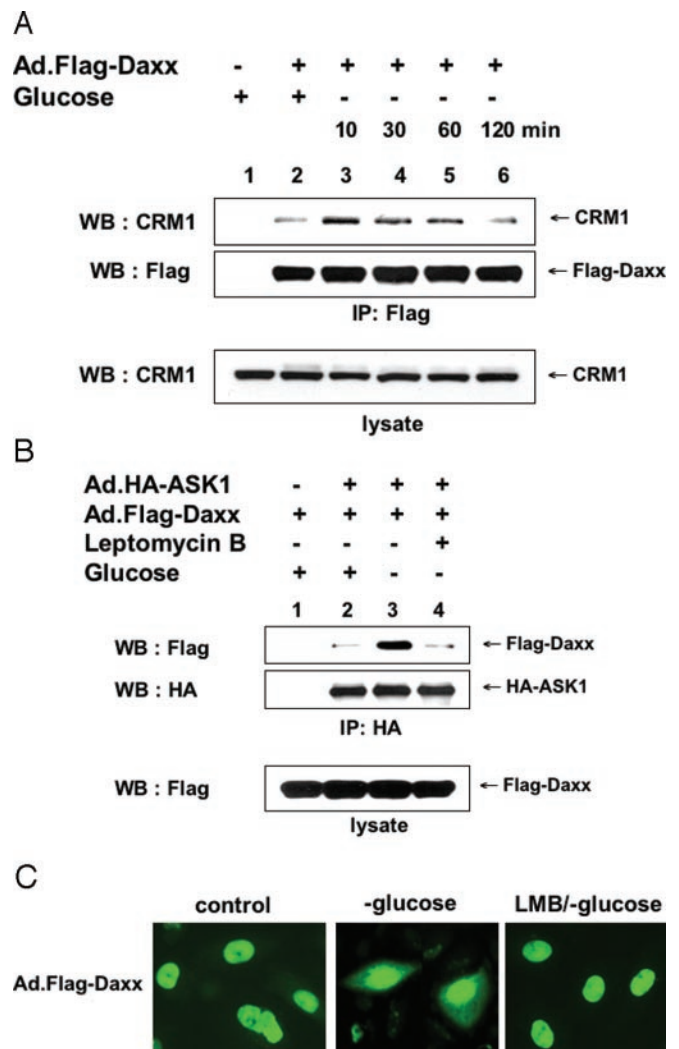
XL1-Blue supercompetent cells. Colonies were selected, and each plasmid (pAdlox-FlagDaxx) was digested with HindIII/KpnI. Its fragment containing the mutation site was subcloned into pBluescript SK(-). Each pBluescript SK(-)-Daxx fragment was sequenced using T7 primer to confirm mutation. pFlagDaxx S667A and S670A were constructed as described previously (6).

**Labeling and Gel Electrophoresis**—To investigate the phosphorylation of Daxx during glucose deprivation, DU-145 cells were transfected with pFlagCMV2-Daxx, pFlagCMV2-Daxx-S667A, or pFlagCMV2-Daxx-S670A. Transfections were done by using LipofectAMINE reagent (Invitrogen). After 48 h of transfection, the cells were pre-equilibrated in phosphate-free medium for 3 h prior to the addition of 100  $\mu$ Ci/ml [<sup>32</sup>P]orthophosphate (PerkinElmer Life Sciences) 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 dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protein inhibitor mixture solution (Sigma). The cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse anti-FLAG 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 dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protein inhibitor mixture solution. The immune complex was separated by SDS-PAGE, and dried gel was visualized by autoradiography.

**Shuttle Vector Construction**—pFlag/CMV2-Daxx was kindly provided by Dr. Horikoshi (Washington University, St. Louis, MO). pAdlox/Flag-Daxx was made by inserting the SpeI/BamHI fragment from pFlag/CMV2-Daxx into the SpeI/BamHI-cut pAdlox shuttle vector (13). Various Daxx deletion mutants, which were FLAG-tagged at their N terminus and had restriction enzyme recognition sites at the flanking sides (5'-EcoRI, 3'-BamHI), were produced by PCR. For Daxx1-625 (amino acids 1-625), the sense primer was 5'-GAGCGAATTCAGCCACCCTAACAGCATCATC-3', and the antisense primer was 5'-CTGCGGATCCCTAACAGCAATCTCCCCAGTTGTG-3'. For Daxx1-500 (amino acids 1-500), the sense primer was the same as that of Daxx1-625, and the antisense primer was 5'-CTATGGATCCCTACTGTAGTGAGGACATGGGGCTC-3'. For Daxx626-739 (amino acids 626-739), the sense primer was 5'-GGTTGAATTCACCCCTGCAA- AAATCTCGG-3', and the antisense primer was 5'-CTATGGATCCCTAATCAGAGTCTGAGAGCAC-3'. 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.

**Adenoviral Vector Construction**—All of the recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system (13). The selective cell line CRE8 has a  $\beta$ -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  $\mu$ g of SfiI/ApaI-digested Adlox/HA-ASK1 fragment or SfiI-digested Adlox/Flag-Daxx and 2  $\mu$ g of  $\psi$ 5 viral genomic DNA were co-transfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and  $\psi$ 5 viral DNA. The new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of HA-ASK1 or FLAG-Daxx to the adenovirus was confirmed by Western blot analysis after infection of the corresponding recombinant adenovirus into DU145 cells.

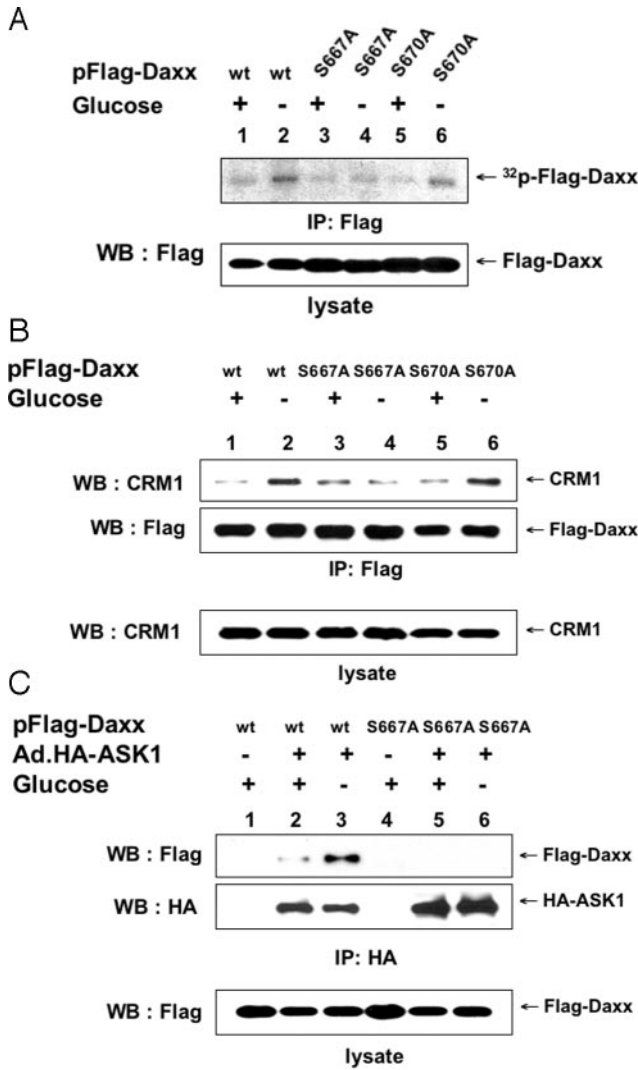
**In Vivo Binding of Daxx with CRM1 or ASK1**—To examine the interaction between Daxx and CRM1, adenovirus of FLAG-tagged Daxx (Ad.Flag-Daxx) at an m.o.i. of 10 was infected into DU-145 cells in 100-mm culture plates. To examine the interaction between ASK1 and Daxx, adenovirus of HA-tagged ASK1 (Ad.HA-ASK1) and FLAG-tagged Daxx (Ad.Flag-Daxx) at an m.o.i. of 10 were co-infected into DU-145 cells in 100-mm culture plates. For immunoprecipitation, after 48 h of infection, the 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 NaOVO<sub>4</sub>, 10 mM  $\beta$ -glycerophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 80  $\mu$ M aprotinin, and 2 mM leupeptin, and the lysates were incubated with 3  $\mu$ g of anti-FLAG M2 mouse IgG1 (Sigma) or 0.5  $\mu$ g of rat anti-HA (clone 3F10, Roche Applied Science) for 2 h, respectively. After the addition of protein G-agarose (Santa Cruz Biotechnology, 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 mouse anti-CRM1 antibody, mouse anti-HA (clone 12CA5, Roche Applied Science) anti-



**FIG. 1. Interaction between Daxx and CRM1 during glucose deprivation.** A, DU-145 cells were infected with adenoviral vector containing FLAG-tagged Daxx (Ad.Flag-Daxx) at an m.o.i. of 10. After 48 h of infection, the cells were exposed to glucose-free medium for various times (10–120 min). The lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted (WB, Western blot) with anti-CRM1 or anti-FLAG antibody (upper panels). The presence of CRM1 in the lysates was verified by immunoblotting with anti-CRM1 antibody (lower panel). IP, immunoprecipitant. B, DU-145 cells were co-infected with adenoviral vector containing HA-tagged ASK1 (Ad.HA-ASK1) at an m.o.i. of 10 and Ad.Flag-Daxx at an m.o.i. of 10. After 48 h of infection, the cells were pretreated with leptomycin B (20 nM) for 4 h and then exposed to glucose-free medium for 1 h. The lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody (upper panels). The presence of FLAG-Daxx in the lysates was verified by immunoblotting with anti-FLAG antibody (lower panel). C, DU-145 cells were infected with Ad.Flag-Daxx at an m.o.i. of 10. After 48 h of infection, the cells were treated with leptomycin B (LMB) (at 20 nM) for 4 h prior to exposure to glucose-free medium for 1 h. The subcellular localization of Daxx was examined by immunofluorescent staining with anti-FLAG antibody.

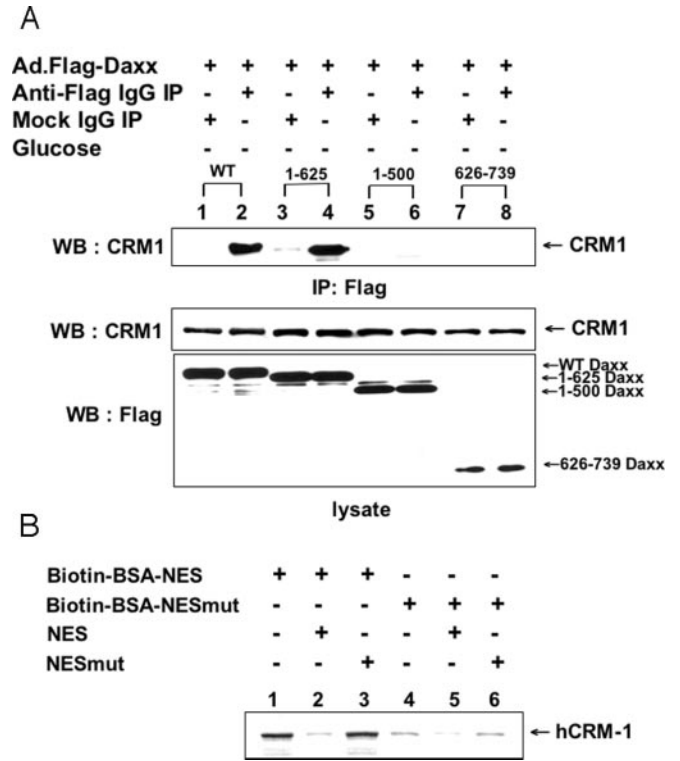
bodies, or mouse anti-FLAG. Proteins in the membranes were then visualized using the ECL reagent as recommended by the manufacturer (Amersham Biosciences).

**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 (phosphate-buffered saline containing 0.1% Tween 20, v/v) and then reacted with primary antibodies. After washing three times with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG. Proteins in the membranes were then visualized using the enhanced ECL reagent (Amersham Biosciences) as recommended by the manufacturer.



**FIG. 2. Phosphorylation of Daxx on Ser-667 and its role in glucose deprivation-induced interaction between Daxx and CRM1.** *A*, DU-145 cells were transfected with pFlagCMV2-Daxx (wild type), pFlagCMV2-Daxx (S667A), or pFlagCMV2-Daxx (S670A) plasmid. After 48 h of transfection, the cells were pre-equilibrated in phosphate-free medium for 3 h prior to the addition of 100  $\mu$ Ci/ml [<sup>32</sup>P]orthophosphate in phosphate- and glucose-free medium for 1 h and lysed. The cell lysates were divided into two portions. One portion was immunoprecipitated (IP) with anti-FLAG antibody, and the immune complex was separated by SDS-PAGE. The dried gel was visualized by autoradiography (upper panel). The other portion was immunoblotted (WB, Western blot) with anti-FLAG antibody (lower panel). *B*, DU-145 cells were transfected with pFlag-Daxx (wild type), pFlag-Daxx (S667A), or pFlag-Daxx (S670A). After 48 h of transfection, the cells were exposed to glucose-free medium for 10 min. The lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-CRM1 or anti-FLAG antibody (upper panels). The presence of CRM1 in the lysates was verified by immunoblotting with anti-CRM1 antibody (lower panel). *C*, DU-145 cells were transfected with pFlag-Daxx (wt, wild type) or pFlag-Daxx (S667A), and infected with Ad.HA-ASK1 at an m.o.i. of 10. After 48 h of incubation, the cells were exposed to glucose-free medium for 1 h. The lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG or anti-HA antibody (upper panels). The presence of FLAG-Daxx in the lysates was verified by immunoblotting with anti-FLAG antibody (lower panel).

**Immunofluorescence**—Cellular localization of FLAG-Daxx was investigated using fluorescence microscopy. The cells were plated onto Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) and infected with Ad.Flag-Daxx at an m.o.i. of 10. After 48 h of infection, the cells were fixed in 100% cold methanol for 10 min at  $-20^{\circ}\text{C}$ . After washing twice with cold PBS, the cells were blocked in 1% BSA plus 10% rabbit serum for 1 h at room temperature. They were then incubated with anti-FLAG (clone M2; mouse) antibody containing 1% BSA plus 10% rabbit serum



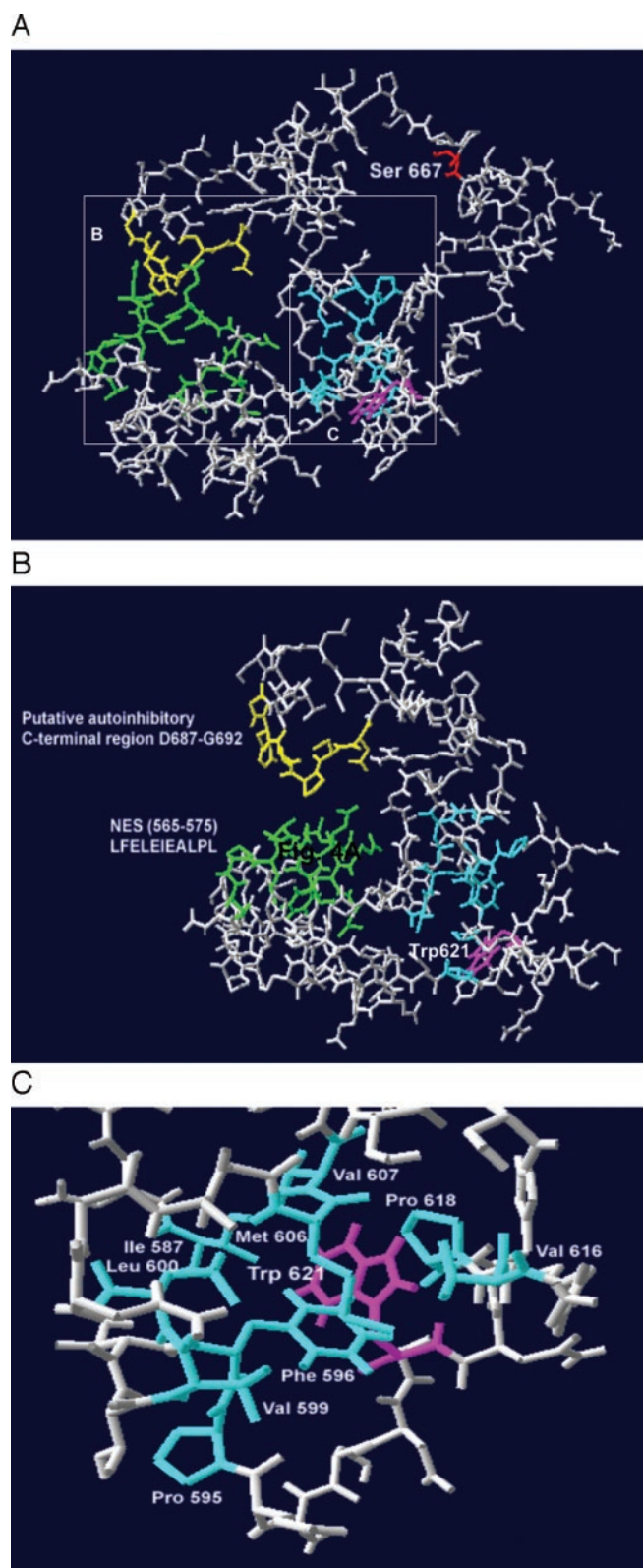
**FIG. 3. Daxx binding site with CRM1.** *A*, DU-145 cells were infected with adenoviral vectors containing FLAG-tagged wild-type Daxx (WT) or various deletion mutant types of Daxx (amino acids 1–625, 1–500, or 626–739) at an m.o.i. of 10. After 48 h of infection, the cells were exposed to glucose-free medium for 10 min. The lysates were immunoprecipitated (IP) with anti-FLAG or mock antibody, and immunoblotted (WB, Western blot) with anti-CRM1 antibody (upper panel). The presence of CRM1 or FLAG-Daxx in the lysates was verified by immunoblotting with anti-CRM1 or anti-FLAG antibody (lower panels). *B*, coupled transcription-translation was performed with the TNT system. [<sup>35</sup>S]Methionine-labeled CRM1 translated *in vitro* was incubated with streptavidin-agarose beads bound to biotinylated BSA-NES (biotin-BSA-NES) or mutated NES (biotin-BSA-NESmut) conjugates. The binding was performed with (+) or without (–) NES or mutated NES (NESmut) peptides (each 2 mg/ml). Bound CRM1 was analyzed by 8% SDS-PAGE and autoradiography. hCRM-1, human CRM-1 protein.

for 1 h at room temperature followed by three washes with cold PBS. Samples of FLAG-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.

**Coupled Transcription-Translation**—For coupled transcription-translation, pXHC1 containing the human CRM1 cDNA, kindly provided by Dr. Minoru Yoshida (RIKEN, Hirosawa, Japan), was digested with XhoI and NotI and then subcloned into XhoI/NotI-cut pTnT<sup>TM</sup> vector. Coupled transcription-translation was performed with the TNT system in reticulocyte lysate (Promega, Madison, WI) supplemented with [<sup>35</sup>S]methionine (Amersham Biosciences catalog no. AG1094). Also, we obtained putative NES or mutated NES affinity columns by coupling 4 mg of biotinylated BSA (Pierce) to 1 mg of sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce). After removal of excess Sulfo-SMCC using a desalting column, the putative NES peptide (LFELEIEALPL) or mutated NES peptide (AFEAEIEAAPA) was incubated with the maleimide-activated biotinylated BSA at 4  $^{\circ}\text{C}$  for 4 h. Biotinylated BSA coupled to the peptides was bound to 30  $\mu$ l of streptavidin-agarose beads (Pierce). After being washed in PBS, the beads were incubated in 30  $\mu$ l of PBS containing BSA (100  $\mu$ g/ml), 5  $\mu$ l of the TNT reaction, and peptides (2 mg/ml) for the competition experiments for 1 h at room temperature. After washing three times with PBS, the beads were treated with Laemmli sample buffer for 5 min in boiling water, and then the samples were analyzed by 8% SDS-PAGE and autoradiography.

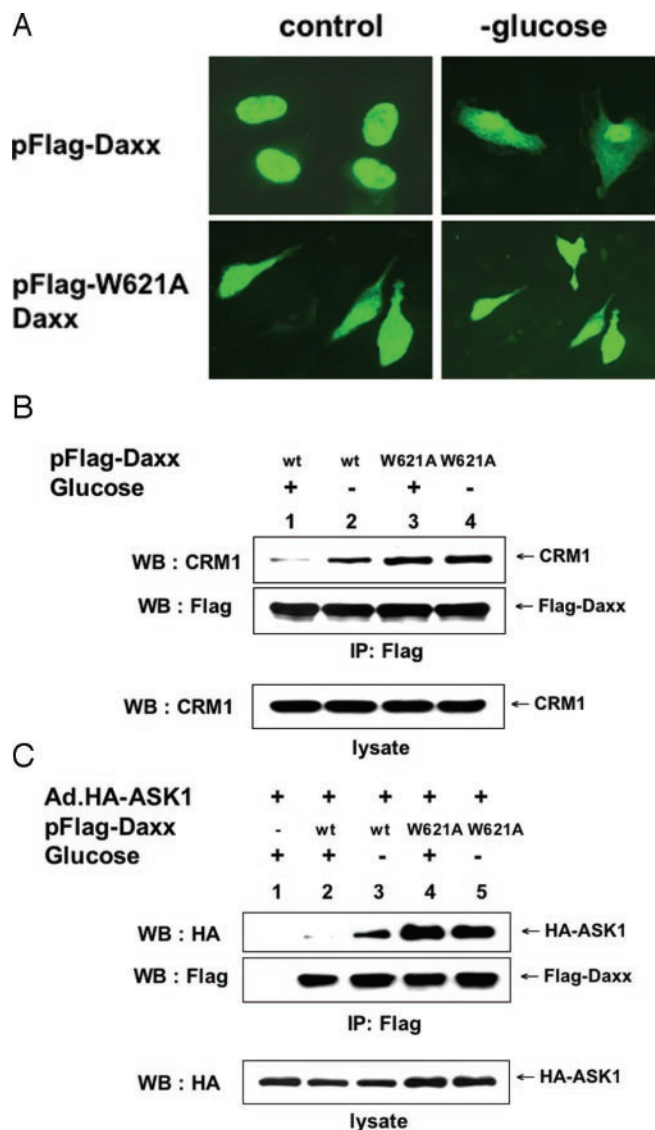
RESULTS AND DISCUSSION

**Daxx Binds to CRM1, and Leptomycin B Inhibits Its Binding to CRM1**—Several researchers reported that CRM1, a member



**FIG. 4. A putative molecular modeling of Daxx.** A primary structure alignment and a putative three-dimensional structure were constructed by using Robetta as a full-chain protein structure prediction server. The different colors help to identify the C-terminal region (yellow), NES (green), hydrophobic region (blue), Ser-667 (red), and Trp-621 (pink). B and C, closer views with labeled side chains using the same coloring mode as in A.

of the  $\beta$ -karyopherin family, mediates nuclear export of several proteins, including human immunodeficiency virus type-1 regulator of expression of virion genes, transcription factor IIIA,

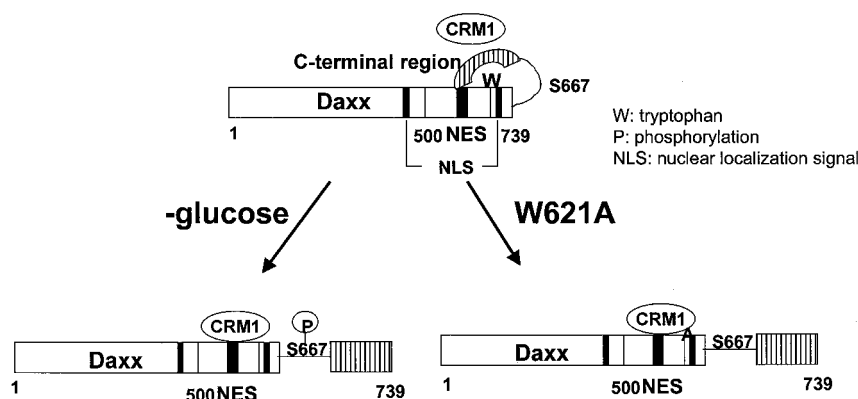


**FIG. 5. Role of tryptophan 621 residue of Daxx in masking the NES from CRM1.** A, DU-145 cells were transfected with either pFlag-Daxx (wild-type) or pFlag-W621A Daxx (mutant type). After 48 h of transfection, the cells were exposed to glucose-free medium for 1 h. The subcellular localization of Daxx was determined by immunofluorescent staining with anti-FLAG antibody. B, DU-145 cells were transfected with pFlag-Daxx (wt, wild type) or pFlag-Daxx (W621A, mutant type). After 48 h of incubation, the cells were exposed to glucose-free medium for 10 min. The lysates were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (WB, Western blot) with anti-CRM1 or anti-FLAG antibody (upper panels). The presence of CRM1 in the lysates was verified by immunoblotting with anti-CRM1 antibody (lower panel). C, DU-145 cells were transfected with pFlag-Daxx (wt, wild type) or pFlag-Daxx (W621A, mutant type) and infected with Ad.HA-ASK1 at an m.o.i. of 10. After 48 h of transfection, the cells were exposed to glucose-free medium for 1 h. The lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-HA or anti-FLAG antibody (upper panels). The presence of HA-ASK1 in the lysates was verified by immunoblotting with anti-HA antibody (lower panel).

protein kinase A inhibitor, and yeast AP-1-like transcription factor (Yap1p) (14–17). These proteins contain a NES that consists of several leucine residues with uneven spacing. We hypothesized that transport of Daxx across the nuclear envelope is mediated through CRM1 and that CRM1 binds to a NES in Daxx and promotes Daxx translocation from the nucleus.

During the first step in this study of the export of Daxx, we investigated whether CRM1 is the binding protein of Daxx during glucose deprivation. Fig. 1A shows that Daxx rapidly bound to CRM1 within 10 min during glucose deprivation.

FIG. 6. A model for the translocation of Daxx through CRM1 during glucose deprivation.



However, the interaction between Daxx and CRM1 gradually decreased as a function of time. We next investigated whether the inhibition of CRM1 prevents the relocalization of Daxx. Previous studies show that a *Streptomyces*-derived antibiotic, leptomycin B, can specifically block CRM1-mediated nuclear export by binding to CRM1 (11, 18). Pretreatment with leptomycin B inhibited the interaction between Daxx and CRM1 as well as relocalization of Daxx during glucose deprivation (Fig. 1, B and C). Taken together, these results demonstrate that glucose deprivation-induced Daxx translocation is mediated through the Daxx-CRM1 interaction. As described previously, ASK1 binds Daxx in the cytosol after a glucose deprivation-induced translocation of Daxx out of the nucleus (6). Leptomycin B blocks translocation of Daxx out of the nucleus (as expected if CRM1 is involved in export) in the absence of glucose and consequently the Daxx-ASK1 interaction. Previous studies have already shown that CRM1, as well as two other proteins, CAS (cellular apoptosis susceptibility) and exportin(tRNA), are involved in exporting proteins from the nucleus. Each nuclear exporter binds to its respective substrates. For example, Yap1p is a target of CRM1 (17). CAS is known to be responsible for importin- $\alpha$  re-export (19). Exportin(tRNA) exports tRNA (20). Recognition of these proteins by the nuclear export receptors occurs in the presence of RanGTP, and in each case a cooperative complex is formed between them (17, 19, 20). Interestingly, oxidative stress inhibits the interaction of CRM1 with Yap1p and subsequently prevents nuclear export of Yap1p. This inhibition requires at least one of the three cysteine residues flanking the NES. These results suggest that a single cysteine residue within the cysteine-rich domain is sufficient to confer redox-sensitive binding to CRM1 (17). However, unlike Yap1p, Daxx is exported from the nucleus to the cytoplasm during oxidative stress (6) (Fig. 1C). These results indicate that a stress-regulated signal for nuclear export is not the same in all cases.

**The Serine 667 Site of Daxx Is Responsible for Its Binding to CRM1**—We reported previously that mutation of Daxx Ser-667 to Ala (S667A) results in suppression of Daxx relocalization during glucose deprivation (6). We postulate that Ser-667 is phosphorylated during glucose deprivation, and its phosphorylation promotes the interaction between Daxx and CRM1. Fig. 2A shows that glucose deprivation increased the phosphorylation of wild-type Daxx and S670A mutant-type Daxx but not Ser-667A mutant-type Daxx. These results indicate that Ser-667 is phosphorylated during glucose deprivation. Fig. 2B shows that, unlike wild-type Daxx and S670A mutant-type Daxx, S667A mutant-type Daxx was not increasingly bound to CRM1 during glucose deprivation. Fig. 2C shows that glucose deprivation increased association between wild-type Daxx and ASK1, which is mainly located in the cytoplasm. In contrast to wild-type Daxx, S667A mutant-type Daxx did not bind to ASK1 during glucose

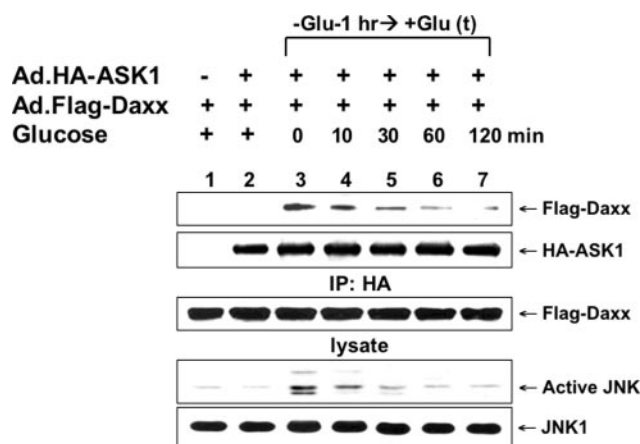


FIG. 7. Effect of glucose replacement on glucose deprivation-induced Daxx and ASK1 interaction and JNK activation. DU-145 cells were co-infected with Ad.HA-ASK1 at an m.o.i. of 10 and Ad.Flag-Daxx at an m.o.i. of 10. After 48 h of infection, the cells were exposed to glucose-free medium for 1 h and then incubated with glucose-containing medium for the indicated times. The lysates were immunoprecipitated (IP) 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 (*middle panel*). Western blot analysis was also done with anti-ACTIVE JNK or anti-JNK1 antibodies.

deprivation. These results suggest that phosphorylation on Ser-667 of Daxx is a prerequisite for interaction with CRM1, Daxx translocation, and subsequent binding to ASK1.

**Daxx565–575 Is the Binding Motif to CRM1**—Next, we attempted to determine which domain of Daxx is responsible for the interaction with CRM1. Cells were infected with adenoviral vectors containing wild-type Daxx or its various Daxx-deletion mutants (amino acids 1–625, 1–500, 626–739). In this study, a Daxx deletion mutant (amino acids 501–625) could not be used, because it is exclusively localized to the cytoplasm (6). Fig. 3A shows that wild-type Daxx and one Daxx deletion mutant (amino acids 1–625), but not other types of mutants (amino acids 1–500 and amino acids 626–739), interacted with CRM1 during glucose deprivation, suggesting that the Daxx binding site for CRM1 exists between amino acids 501–625. As mentioned previously, Daxx contains a putative NES (amino acids 565–575). We postulated that the putative NES of Daxx interacts with CRM1. To test this possibility, we conjugated biotinylated BSA to Daxx NES (LFELEIEALPL) or mutated NES (AFEAEIEAAPA) peptides and coupled it to streptavidin-agarose. CRM1 translated *in vitro* bound to a NES affinity column. However, little binding of CRM1 was observed when leucine residues of NES were replaced with alanines (Fig. 3B). CRM1-NES interaction was prevented by the addition of an excess of NES peptide but was not affected by the mutated NES (Fig. 3B,

lane 2 versus lane 3). These results demonstrate that the NES of Daxx is a binding site for CRM1.

**Tryptophan Residue at 621 of Daxx Acts as an Inhibitory Residue Masking the NES**—A fundamental question, which remains unanswered, is why phosphorylation on Ser-667 of Daxx is required for interaction between Daxx and CRM1. Molecular modeling was used to investigate the role of phosphorylated Ser-667 in the interaction of Daxx with CRM1. On the basis of a primary structure alignment and a putative three-dimensional structure of the C-terminal region and the NES domain, we speculated that Daxx contains an autoinhibitory A-helix motif at the C-terminal region (Fig. 4). A tryptophan residue at position 621 may interact by van der Waals contacts with the hydrophobic region in the C terminus and consequently prevent interaction between the NES of Daxx and CRM1. Phosphorylation of Ser-667 results in a negative phosphate charge and subsequently disrupts the interaction between the tryptophan side chain of the A-helix and the hydrophobic region of the C terminus. This disruption allows CRM1 to bind to the NES of Daxx. To test our hypothesis, we replaced the tryptophan at position 621 of Daxx with alanine (W621A). Fig. 5A shows that wild-type Daxx was relocalized from the nucleus to the cytoplasm during glucose deprivation (*top panels*). However, mutant-type Daxx (W621A) was not confined within the nucleus regardless of glucose deprivation (Fig. 5A, *bottom panels*). CRM1 and ASK1 increasingly bound to mutant-type Daxx (W621A) regardless of glucose deprivation (Fig. 5, *B and C*). These results and molecular modeling of Daxx (Fig. 6) illustrate that the Trp-621 could fit into the hydrophobic pocket of the C terminus resulting in prevention of the interaction between CRM1 and Daxx. Phosphorylation at serine 667 probably unmasks the NES of Daxx to CRM1 by destabilizing the inhibitory motif. Similar observations were reported in cyclin B1, mitogen-activated protein kinase (MAPK)-activated protein kinase 2, p27<sup>Kip1</sup>, and snail gene product (12, 21–23). Phosphorylation of each of these is required for binding to CRM1 and nuclear export. For example, phosphorylation of mitogen-activated protein kinase (MAPK)-activated protein kinase 2 at Thr-317 may destabilize the A-helix itself and/or its binding to the hydrophobic cleft and consequently release the autoinhibitory motif. This process contributes to activation of the enzyme and, in parallel, might unmask the NES (24). As a result, the activated kinase is exported rapidly from the nucleus to the cytoplasm.

**Involvement of Daxx in ASK1-mediated JNK Activation**—We observed previously that glucose deprivation activates the ASK1-SEK1-JNK1-HIPK1 pathway, and the activated HIPK1 phosphorylates Daxx. The phosphorylated Daxx is translocated to the cytoplasm (as discussed in the preceding paragraphs), binds to ASK1, and subsequently leads to ASK1 oligomerization. We hypothesized that association of Daxx with ASK1 maintains JNK activity. To examine this possibility, DU-145 cells were exposed to glucose-free medium for 1 h and then incubated with complete medium for various times (0–120 min). Fig. 7 shows that glucose deprivation-induced association

of Daxx with ASK1 as well as JNK activity gradually reduced during incubation with complete medium. These results suggest that Daxx is involved in the ASK1-JNK1 activity. At this time, only speculation can be made concerning the biological function of relocalized Daxx during glucose deprivation. Several researchers have shown that Daxx acts as a transcriptional regulator in the nucleus (25–27). However, its transcriptional activity is relieved when Daxx relocalizes to the cytoplasm (28). Our studies demonstrate that cytoplasmic Daxx binds to ASK1 and is subsequently involved in the oligomerization of ASK1. Although we are far from understanding the role of Daxx in the cytoplasm, we speculate that ASK1 oligomerization maintains ASK1 activity by preventing ASK1 degradation. We believe that this model will provide a framework for future studies.

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