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Physical Interactions and Functional Coupling between Daxx and Sodium Hydrogen Exchanger 1 in Ischemic Cell Death*[S]

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Daxx, a death domain-associated protein, is implicated in ischemic cell death. To clarify the mechanism of cell death mediated by Daxx, a yeast two-hybrid assay was performed. Sodium hydrogen exchanger isoform 1 (NHE1) was identified as a Daxx-interacting protein. During ischemic stress, Daxx translocates from the nucleus to the cytoplasm, where it colocalizes with NHE1. Daxx binds to the ezrin/radixin/moesin-interacting domain of NHE1, in competition with ezrin. Consistent with this finding, transfection of the constitutively cytoplasmic mutant, Daxx(W621A), inhibited ezrin-mediated Akt-1 activation. Moreover, transfection of Daxx(W621A), but not the Daxx(S667A) mutant that is confined to the nucleus, accelerated pH_i recovery from an acid load, indicating that the cytoplasmic protein activates NHE1. Based on the results, we propose that ischemic insult triggers the nucleocytoplasmic translocation of Daxx, following which cytoplasmic Daxx stimulates the NHE1 transporter activity and suppresses activation of the NHE1-ezrin-Akt-1 pathway. Our data support a novel molecular function of Daxx as an upstream regulator of NHE1 in ischemic cell death.

Daxx was initially identified as an interacting protein with the cytoplasmic domain of Fas that potentiates Fas-induced cell death as a signal transducer (1). Daxx activates the apoptosis signal-regulating kinase 1 → c-Jun N-terminal kinase (JNK)[4] pathway upon glucose deprivation (2, 3). In addition to its function as a signal transducer in the cytoplasm, the presence of Daxx in the nucleus has been reported. Specifically, Daxx localizes in the promyelocytic leukemia protein and STAT-3 nuclear bodies (4–9) and exerts strong transcriptional repression (4, 5, 7, 10–14). This discrepancy in the observed subcellular localization of Daxx may be attributed to nucleocytoplasmic translocation, which occurs following Fas stimulation (15), heat shock (16), oxidative stress (17), and ischemic insult (18, 19).

Daxx is involved in stress-induced cell death (16–20). However, the issue of whether Daxx promotes or prevents cell death is currently unresolved. Depletion of Daxx with small interfering RNAs protected cells from interferon γ- and As_2O_3-induced death (21), supporting its role as a death-promoting protein. Furthermore, Daxx overexpression potentiates apoptosis induced by Fas and transforming growth factor-β (1, 22). However, the view that Daxx is a death-promoting protein has often been challenged. For instance, Daxx knockout embryos display early embryonic lethality and extensive apoptosis (23). Moreover, Daxx silencing sensitizes cells to Fas-, UV-, and tumor necrosis factor-α-induced cell death (13, 24). It is possible that Daxx performs bipartite functions, depending on the situation.

Sodium hydrogen exchanger (NHE) exchanges one intracellular H^+ ion for an extracellular Na^+ ion (25). NHE1, the first to be cloned among the nine NHE isoforms (26), is ubiquitously expressed in various cell types, and acts as a "housekeeping" protein involved in the maintenance of pH homeostasis and cell volume regulation. The N terminus contains 12 transmembrane domains and retains ion exchange activity, whereas the C terminus modulates NHE1 activity through phosphorylation and binding to other proteins. The C-terminal region of NHE1 is phosphorylated by p90RSK, Nck-interacting kinase, protein kinases A and C, p160ROCK, and p38 (27–31). Interactions with proteins devoid of kinase activity have additionally been reported. Calcineurin homologous protein and tescalcin bind to NHE1 and inhibit the transporter (32–34), whereas binding of carbonic anhydrase II and calmodulin activates NHE1 (35–37). Moreover, NHE1 participates in cell signaling. Studies show that NHE1 binds to the ezrin/radixin/moesin (ERM) complex (38, 39) and activates the ERM → PI3K → Akt pathway (40–42).

NHE1 involvement in ischemic damage is evident in cardiac myocytes and primary neurons (43–48). Following intracellular acidosis during ischemia (49), NHE1 activation may be ben-

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[4] The abbreviations used are: JNK, c-Jun N-terminal kinase; NHE, sodium hydrogen exchanger; ERM, ezrin/radixin/moesin; GST, glutathione *S*-transferase; CHP, calcineurin homologous protein; CH, chemical hypoxia; PBS, phosphate-buffered saline; pH_i, intracellular pH; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; DIC, differential interference contrast; NHE1cd, cytoplasmic domain of NHE1; STAT-3, signal transducers and activators of transcription 3; PI3K, phosphatidylinositol 3-kinase; TRITC, tetramethylrhodamine isothiocyanate.

eficial in restoring the intracellular pH by extruding H^+ to a certain extent (50, 51). However, overstimulation of NHE1 enhances the intracellular Na^+ concentration, which, in turn, reverses the driving force for the Na^+/Ca^{2+} exchanger, resulting in elevation of intracellular Ca^{2+}. This excessive Ca^{2+} influx leads to cell death (52). In fact, NHE1$^{-/-}$ neurons exhibit resistance to ischemia, both *in vitro* and *in vivo* (53). Pharmacological inhibitors against NHE1 are currently under development for therapeutic use under the premise that suppression of NHE1 would interrupt intracellular Ca^{2+} elevation and protects cells upon ischemic insult (54).

In the present study, we elucidate the molecular mechanism of Daxx-mediated cell death during ischemic insult. NHE1 is identified as a Daxx-interacting molecule. Our results demonstrate that the nucleocytoplasmic translocation of Daxx facilitates a death-promoting role through activation of the NHE1 transporter and suppression of NHE1-associated Akt-1 activity under conditions of ischemic stress.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—LexA/Daxx constructs were generated using a human HeLa cDNA library as a template and PCR primers containing EcoRI and XhoI restriction sites as previously described (55). The generated fragments were cloned into the EcoRI/XhoI restriction sites of pLexA in-frame with the LexA DNA-binding domain. The pFlag/Daxx(W621A) and pFlag/Daxx(S667A) constructs were kindly provided by Drs. Song and Lee (University of Pittsburgh, Pittsburgh, PA). Plasmids encoding glutathione *S*-transferase (GST)-Daxx fusion proteins and Daxx deletion fragments (amino acids 1–130, 131–400, 400–500, 500–625, and 625–740) were generated by PCR from full-length Daxx, followed by cloning into pGEX-4T-1 vector (Amersham Biosciences). The NHE1 deletion fragments (cytoplasmic domain (cd) (amino acids 503–815), calcineurin homologous protein (CHP)-binding domain (amino acids 503–567), ERM-binding domain (amino acids 567–637), calmodulin-binding domain (amino acids 637–691), and kinase domain (amino acids 691–815)) were generated by PCR using the primers having restriction enzyme sites at the flanking sides (5′, EcoRI; 3′, XhoI) and cloned into EcoRI/XhoI-cut pGEX-4T-1. GST fusion proteins were purified by affinity chromatography using glutathione-Sepharose 4B (Peptron Inc., Daejeon, Korea). Full-length NHE1 and Daxx cDNA sequences were cloned into pcDNA3 vector (Invitrogen). Full-length ezrin was ligated within the EcoRI/XhoI sites of pcDNA3 (Invitrogen Inc.).

Yeast Two-hybrid Screening—pLexA/Daxx was used to screen a human HeLa cDNA library (Clontech, Palo Alto, CA). The bait plasmid was introduced into the yeast strain, EGY48, using the lithium acetate-mediated method. Yeast expressing the bait protein was sequentially transformed with a human HeLa cDNA library constructed in pB42AD (Clontech) and plated on Synthetic drop-out agar deficient in leucine, tryptophan, and histidine. Approximately 5.5×10^5 transformants of the EGY48 strain were screened using the manufacturer's protocols. In general, colonies grew in 3–6 days and were screened for β-galactosidase activity. Library plasmids from strongly interacting clones were isolated and retransformed into the reporter strain to examine for interactions with Daxx, along with positive or negative control baits.

Cell Culture and DNA Transfection—Parental Chinese hamster lung fibroblast CCL39, NHE-deficient PS120 (50), NHE1-expressing PS120/NHE1 (56), BOSC23 and HeLa cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 1% antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. PS120/NHE1 cells (1×10^6) were seeded at 50% confluence onto 60-mm dishes 24 h prior to transfection. Next, cells were transfected with 2 μg of plasmid DNA using METAFECTENE (Biontex, Munich, Germany), according to the manufacturer's protocols. After 3 h of incubation with DNA-lipid complexes, cells were refed with serum-containing medium. BOSC23 cells (1×10^6) were pre-cultured for 12 h and subsequently transfected with the indicated plasmids, using the calcium phosphate method. Transiently transfected cells were harvested after 48 h.

Simulations of Ischemia—To induce conditions of chemical hypoxia (CH), cells were washed once with phosphate-buffered saline (PBS) and placed in the metabolic inhibition buffer (106 mM NaCl, 4.4 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 38 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 20 mM 2-deoxy-D-glucose, 1 mM NaCN, pH 6.6) for the indicated time periods (57). For glucose deprivation, cells were exposed to glucose-free medium for 2 h and split (1:5) at 80–90% confluence. In the case of using an anaerobic chamber, cells were incubated in serum-free, glucose-free Dulbecco's modified Eagle's medium using an anaerobic chamber (Forma Scientific, Marietta, OH) at 37 °C with 5% CO_2, 10% H_2, and 85% N_2 (19).

In Vitro Binding of Daxx with NHE1—To analyze the interactions between Daxx with NHE1, GST fusion proteins were expressed in *Escherichia coli* BL21(DE3) induced with isopropyl-β-D-thiogalactopyranoside. Subsequently, cells were sonicated in ice-cold lysis buffer (200 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 100 μM EDTA, 0.1% Triton X-100, 0.4 mM phenylmethylsulfonyl fluoride). GST fusion proteins were incubated with glutathione-coated beads for 4 h at 4 °C and normalized for the protein concentration. pcDNA3/Daxx and pcDNA3/NHE1cd were translated *in vitro* with TNT® Quick Coupled Transcription/Translation Systems (Promega Corp., Madison, WI). Briefly, 2 μg of DNA was incubated with 10 μCi of [^{35}S]methionine in TNT® Quick Master mix for 90 min at 30 °C. *In vitro* translated products were mixed with GST-fused proteins bound to glutathione-coated beads in E1A binding buffer (50 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol), and incubated for 4 h at 4 °C. After washing three times in E1A binding buffer, samples were treated with SDS-sample buffer. Subsequently, samples were subjected to SDS-PAGE, and analyzed by autoradiography.

In Vitro Competition Assay—GST-NHE1cd and GST-Daxx-(1–400) fusion proteins were purified from bacteria using glutathione-Sepharose. Ezrin (10 μl, ^{35}S-labeled) was incubated with resin-bound GST-NHE1cd in the presence of non-isotope-labeled Daxx at the indicated volumes for 4 h at 4 °C. The resins were washed with E1A binding buffer, boiled in sample buffer, and analyzed by SDS-PAGE. In another competition experiment, 10 μl of ^{35}S-labeled NHE1cd was incubated with

resin-bound GST-Daxx-(1–400) in the presence of non-isotope labeled ezrin, as described above.

Immunoprecipitation and Western Analysis—Cells were washed with PBS, lysed in mammalian lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.4 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 13,000 rpm for 10 min. For the immunoprecipitation assay, cell lysates were incubated for 4 h at 4 °C with anti-Daxx (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-NHE1 antibodies (58) coupled to the protein A/G-agarose beads (Santa Cruz Biotechnology). A monoclonal M3-anti-FLAG antibody preconjugated to agarose beads was used to immunoprecipitate FLAG-tagged proteins. Immunoprecipitated beads were washed three times with mammalian lysis buffer. Immunoprecipitates and whole cell lysates were subjected to an SDS-PAGE, separated, and transferred to a nitrocellulose membrane. Blots were probed overnight with the appropriate dilutions of primary antibodies at 4 °C. Membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution). Membranes were washed and developed with ECL plus Western blotting detection reagents.

Intracellular pH Measurement—Na^+/H^+ exchange activity was measured using a standard protocol with some modifications (59, 60). Briefly, transfected cells grown on glass coverslips were loaded with a pH-sensitive fluorescent dye, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), and intracellular pH (pH_i) changes were measured. BCECF fluorescence was recorded at excitation wavelengths of 440 and 490 nm using the recording setup (Delta Ram, PTI Inc., Brunswick, NJ). Cells were acidified with an NH_4^+ (20 mM) pulse and subsequent perfusion with Na^+-free solution. Maximal Na^+-dependent pH_i recovery was measured in cells acidified to a pH of 6.4–6.5. The standard perfusion solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). Na^+-free solutions were prepared by replacing Na^+ with *N*-methyl-D-glucamine$^+$. The osmolarity of all solutions was adjusted to 310 mM with the major salt. Buffer capacity was calculated by measuring pH_i in response to 5–20 mM NH_4Cl pulses. In each experiment, the intrinsic buffer capacity (β_i) displayed a negative linear relationship with pH_i between 6.4 and 7.3. The β_i of PS120 cells was 34.1 ± 3.9 mM NH_4^+/pH unit at $pH_i = 7.0$. However, none of the gene alterations significantly altered β_i. NHE1 activity is expressed as ΔpH/min. This value was directly analyzed without compensating for β_i.

Immunofluorescence—For analysis of the subcellular localization of Daxx upon ischemic insult, PS120/NHE1 cells were plated on sterile coverslips in 6-well plates. After CH treatment, cells were fixed in 3.7% formaldehyde for 15 min and 1× PBS, incubated with anti-Daxx (Santa Cruz Biotechnology) and anti-NHE1 antibodies (BD Biosciences, Boston, MA) for 45 min, and washed twice with 1× PBS, followed by treatment with secondary antibodies for 30 min. Cells were rewashed three times with 1× PBS, and mounted on slides. For determining the cellular localization of Daxx and NHE1, PS120/NHE1 cells transfected with FLAG-tagged Daxx mutants were plated on a coverslip. Next, cells were fixed in 3.7% formaldehyde for 15 min and rinsed with 1× PBS. The cells were then incubated with anti-FLAG (Sigma) and anti-NHE1 antibodies for 1 h, washed twice with 1× PBS, and treated with the respective secondary antibodies conjugated to fluorescein isothiocyanate and TRITC (Sigma), for 30 min. After mounting on slides, color images of cells were acquired using an inverted Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany).

Measurement of Cell Death—The 4′,6-diamidino-2-phenylindole staining protocol was modified slightly, as described previously (61). Cells were grown on coverslips in 6-well culture plates. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min, stained with 2 μg/ml of 4′,6-diamidino-2-phenylindole, and counted under a fluorescent microscope. A differential interference contrast (DIC) optical microscope was used to record the images of cells with a charge-coupled device camera (Axiocam MRm, Carl Zeiss) attached to a Zeiss Axiovert microscope. The cytotoxicity was assessed by monitoring lactate dehydrogenase activity in the extracellular medium. lactate dehydrogenase was measured colorimetrically according to the manufacturer's instruction (Promega). The percent lactate dehydrogenase was calculated from the maximum lactate dehydrogenase release (100%) induced by lysing cells with 0.1% Triton X-100.

RESULTS

Identification of NHE1 as a Daxx-binding Protein—To clarify the mechanism of Daxx action in ischemic cell death, a HeLa cDNA library was screened with the yeast two-hybrid system utilizing Daxx as bait. Positive clones were selected based on the expressions of β-galactosidase reporter and *leu* genes, which allowed growth on leucine-deficient plates. DNA sequencing and subsequent GenBank™ searches revealed that three strongly positive clones contained partial sequences of NHE1. Accordingly, NHE1 was selected for further study (data not shown).

The *in vitro* translated cytoplasmic domain of NHE1 (NHE1cd) interacted with the GST-Daxx fusion protein, but not GST alone (Fig. 1*A*, *left*). *In vitro* translated Daxx additionally bound to GST-NHE1cd (Fig. 1*A*, *right*). This association between NHE1 and Daxx was confirmed by coimmunoprecipitation of NHE1 with FLAG-tagged Daxx transfected in BOSC23 cells (supplemental Fig. S1). Our results clearly indicate that Daxx can physically associate with NHE1.

To determine the binding domains in NHE1-Daxx interactions, GST fusions of various deletion mutants of both NHE1 and Daxx were generated (Fig. 1*B*). *In vitro* translated Daxx was incubated with GST-NHE1 mutants (Fig. 1*B*, *left panel*). Daxx bound to NHE1-ERM, but not NHE1-CHP, NHE1-calmodulin, or NHE1-kinase domains. Moreover, *in vitro* translated NHE1 was incubated with GST-Daxx mutants (Fig. 1*B*, *right panel*). Interaction analyses revealed that amino acids 1–400 of Daxx associate with NHE1 (Fig. 1*B*, *right panel*). Endogenous binding was investigated in PS120/NHE1 cells stably transfected with cDNA encoding NHE1 with two experimental simulations of ischemia, CH, and glucose deprivation (Fig. 1*C*). NHE1-Daxx association in quiescent cells was weak, but markedly enhanced in cells stimulated with CH or deprived of glucose. Similarly, we observed increased NHE1-Daxx association upon CH and glucose deprivation in HeLa cells (supplemental Fig. S2).

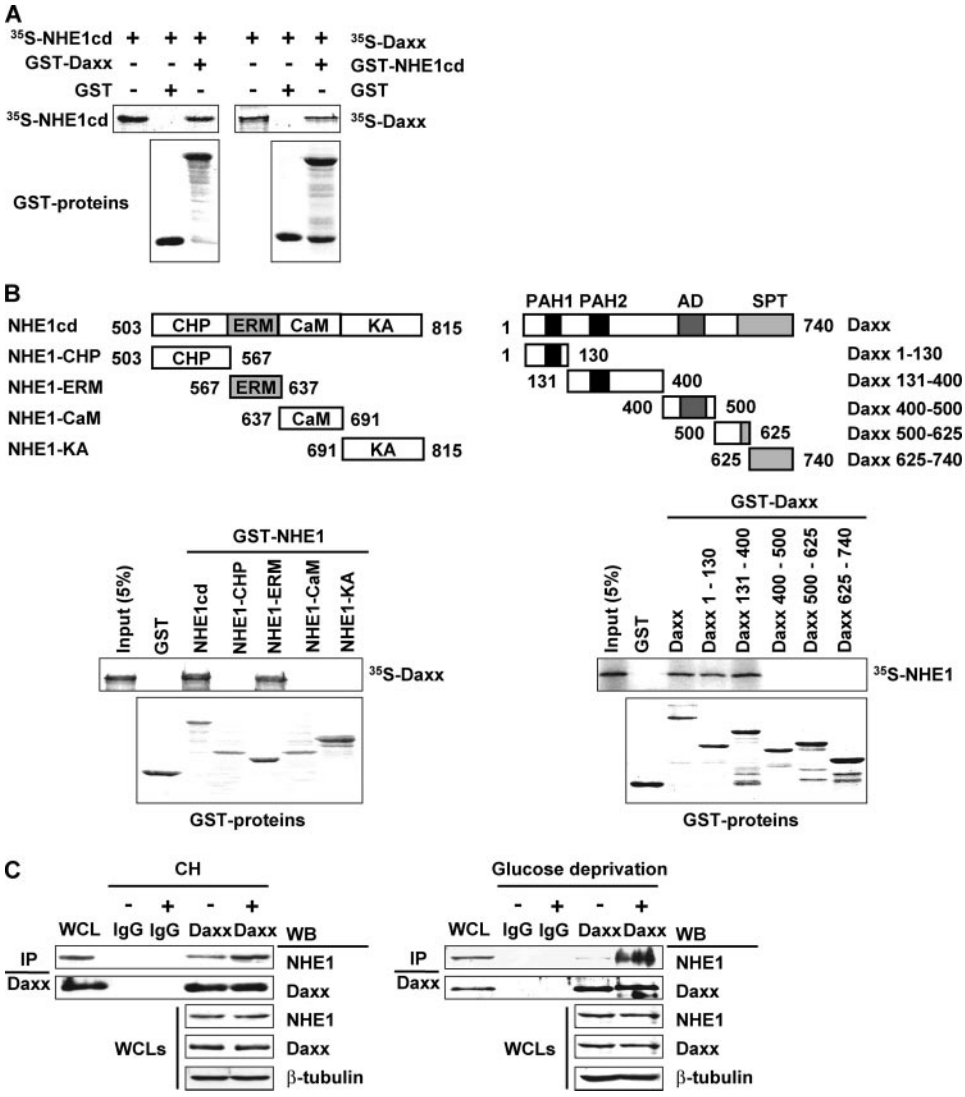

FIGURE 1. **Daxx interacts with NHE1.** *A*, *in vitro* translated ^{35}S-labeled products were incubated with GST, GST-Daxx, or GST-NHE1cd (amino acids 503–815) immobilized on glutathione-Sepharose beads for 4 h. ^{35}S-Labeled bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE for autoradiography. *B*, to identify the region of NHE1 interacting with Daxx, *in vitro* translated ^{35}S-labeled Daxx and NHE1cd-truncated mutants fused to GST were incubated (*left panel*). Conversely, ^{35}S-labeled NHE1 was incubated with GST-Daxx deletion mutants (*right panel*). *Upper panels*, schematic drawing of NHE1cd and Daxx mutant constructs. Coomassie Brilliant Blue-stained GST fusion proteins from the same gel were aligned to estimate protein levels. *C*, PS120/NHE1 cells were subjected to with CH (90 min, *left panel*) or glucose deprivation (120 min, *right panel*). Endogenous Daxx was immunoprecipitated with anti-Daxx or control antibody from PS120/NHE1 cell lysates. Immunoprecipitates were subjected to Western blotting, and blots were probed with anti-NHE1 or anti-Daxx antibody. Whole cell lysates were immunoblotted with anti-NHE1, anti-Daxx, and anti-β-tubulin antibodies. β-Tubulin was used to confirm the amount of protein loaded in each lane. *AD*, acid-rich domain; *SPT*, Ser/Pro/Thr-rich domain; *PAH*, paired amphipathic α helices domain; *WCL*, whole cell lysate; *IP*, immunoprecipitation.

Nuclear Export of Daxx and Subsequent Colocalization with NHE1 under CH Conditions—We examined the subcellular localization of NHE1 and Daxx upon ischemic stimulation under a confocal microscope. Time-course immunofluorescence confocal microscopy revealed that the presence of NHE1 in the cytoplasm and nuclear envelope (Fig. 2*A*, *second row*). In contrast, Daxx appeared as nuclear speckles in resting PS120/NHE1 cells. However, following CH, the majority of Daxx was exported to the cytoplasm of PS120/NHE1 cells (Fig. 2*A*, *top row*) colocalizing with NHE1 (Fig. 2*A*, *third row*). The *graphs* represent fluorescence distributions determined for cell sections (Fig. 2*A*, *bottom row*).

To analyze the correlation between Daxx trafficking and its colocalization with NHE1, two Daxx mutants with different subcellular destinations were employed. FLAG-tagged Daxx(W621A) (18), a mutant that is constitutively expressed in the cytoplasm, was transfected into PS120/NHE1 cells. Daxx(W621A) colocalized with NHE1 in the cytoplasm in the absence of CH (Fig. 2*B*). Conversely, the Daxx(S667A) mutant that is confined to the nucleus did not colocalize with NHE1 (Fig. 2*B*). These data indicate that the subcellular localization of Daxx influences Daxx-NHE1 interactions.

Cytoplasmic Daxx Activates the NHE1 Transporter—To establish the physiological implications of NHE1-Daxx binding, we overexpressed Daxx(W621A) in PS120/NHE1 cells and measured NHE1 activity by determining the rate of intracellular pH recovery from an NH_4Cl-induced acid load (60). NHE1 restores the intracellular pH (pH_i) by extruding protons, and alkalinizes cells (62). The intracellular pH recovery after acid load in Daxx(W621A)-transfected cells was more rapid than that in mock transfected cells, indicating that Daxx(W621A) stimulates NHE1 to extrude H^+ (Fig. 3*A*). The H^+ transport rate in Daxx(W621A)-transfected cells was enhanced to 166 ± 28%, compared with that in mock transfected cells. In contrast, transfection with Daxx(S667A) failed to potentiate NHE1 activity. The pH_i change was abolished in the presence of the NHE1-specific inhibitor, cariporide, signifying that pH_i is caused by NHE1 activation (summarized in Fig. 3*B*). Thus, it appears that direct interactions with Daxx are required to activate NHE1.

Daxx Competes with ERM for Binding to NHE1—Because both Daxx and ERM bind to positions 567–637 of NHE1, we examined whether the two proteins compete for interactions with NHE1 (Fig. 1*B* and supplemental Fig. S3). NHE1-ERM interactions tether NHE1 to the actin cytoskeleton, leading to activation of the PI3K/Akt-1 signaling pathway (38, 39, 63).

The interaction profiles of Daxx, ERM, and NHE1 in response to CH were analyzed using immunoprecipitation assays. Consistent with previous data (38), major interactions were observed between positions 567–637 of NHE1 and *in*

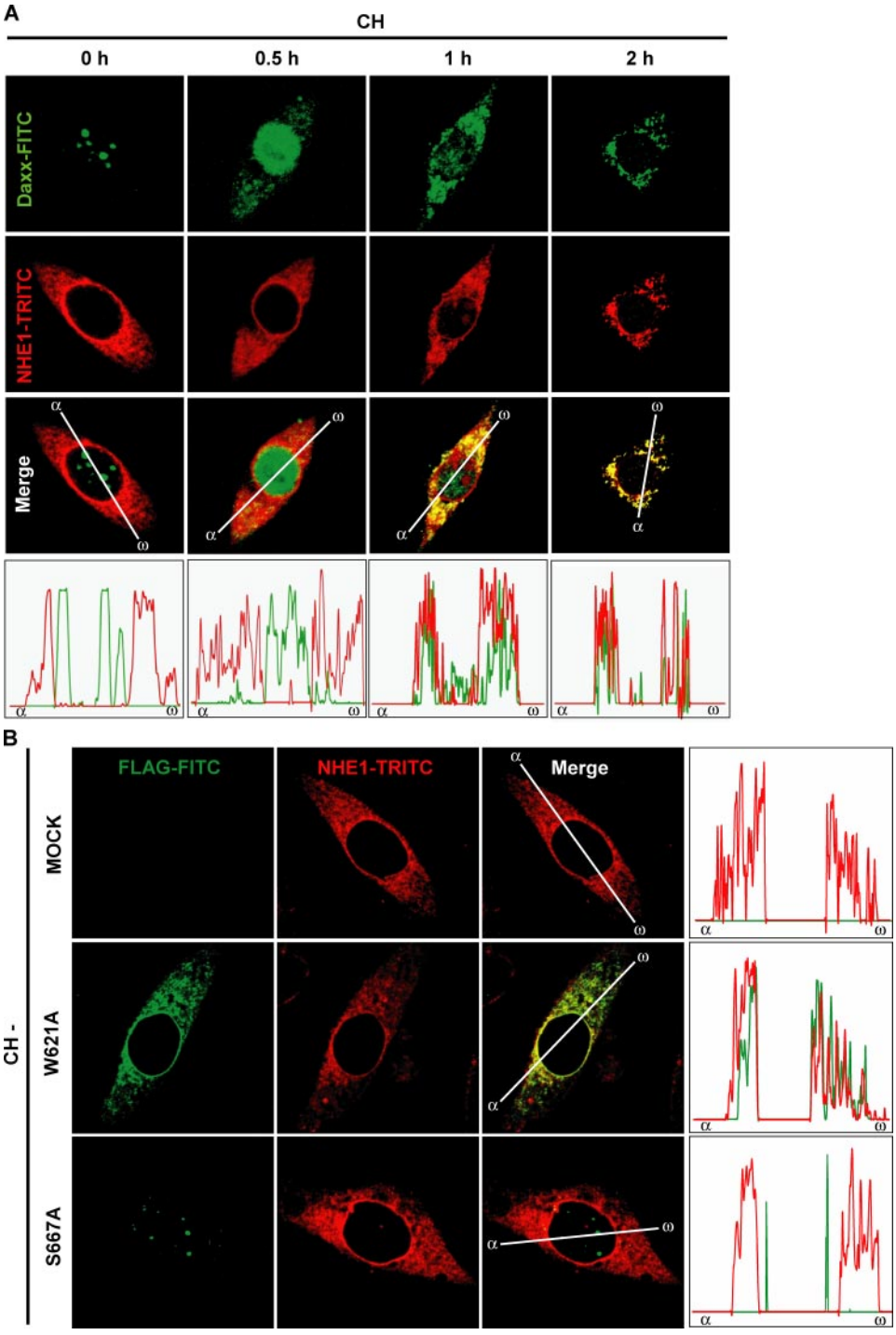

FIGURE 2. **NHE1-Daxx interactions occur following Daxx nuclear export.** *A*, PS120/NHE1 cells were exposed to CH for the indicated periods of time. Representative confocal images and quantitative analysis of subcellular protein distribution (*bottom row*) from PS120/NHE1 cells after CH at the indicated times. The *overlay* represents a fusion image of *green* (Daxx) and *red* (NHE1) fluorescence. *Yellow* reflects colocalization of Daxx and NHE1. *B*, after 48 h of transfection with the indicated plasmids, transfected cells were stained with anti-FLAG antibody (*green*) and anti-NHE1 (*red*). The *graphs* represent fluorescence distribution determined for cell sections, as indicated in the *green/red* fusion image. α and ω signify the orientation of the section. All *panels* show representatives of three independent experiments. *MOCK*, vector only.

vitro translated ezrin (supplemental Fig. S3). However, ezrin bound weakly to the NHE1-CHP domain under our experimental conditions (supplemental Fig. S3). ERM bound to NHE1 in quiescent cells (Fig. 4*A*). After exposure to CH, NHE1 dissociated from ERM, and association of NHE1 with Daxx was increased in PS120/NHE1 cells. Similar binding patterns were observed in HeLa cells (supplemental Fig. S4). To elucidate whether Daxx plays a causative role in the dissociation of ERM from NHE1, we examined whether addition of a cytoplasmic Daxx mutant (W621A) prevents ERM-NHE1 association. Transfection of FLAG-tagged Daxx(W621A) significantly disrupted NHE1-ERM binding without CH (Fig. 4*B*).

To further confirm the competition between Daxx and ERM for NHE1 binding, we performed an *in vitro* protein binding competition assay. Upon incubation of GST-NHE1cd with ^{35}S-labeled ezrin and increasing amounts of non-labeled Daxx, a dose-dependent decrease in interactions between NHE1cd and ezrin was evident (Fig. 4*C*). We additionally used GST-Daxx-(1–400) to pull down ^{35}S-labeled NHE1cd mixed with increasing amounts of non-labeled ezrin. Ezrin inhibited NHE1-Daxx interactions in a dose-dependent manner (supplemental Fig. S5). Our results clearly indicate that Daxx and ERM compete for binding to NHE1.

To establish whether Daxx regulates NHE1-ERM-dependent Akt-1 activation, PS120/NHE1 cells were exposed to CH, and lysates probed for Akt-1 activity with an anti-phospho-Akt-1 antibody. The extent of Akt-1 phosphorylation declined in a time-dependent manner (Fig. 4*D*, *upper panel*), and Akt-1 activity was dramatically suppressed 2 h after CH. Consequently, the Akt-1 phosphorylation experiment was performed 2 h after CH. Akt-1 phosphorylation was enhanced in ezrin-transfected cells, whereas Daxx(W621A)-transfected cells exhibited significantly lower Akt-1 phosphorylation (Fig. 4*D*, *lower panel*). Accordingly, we propose that cytoplasmic Daxx blocks the ERM-mediated cell survival pathway involving Akt-1.

NHE1-Daxx Interactions Mediate CH-induced Cell Death—Excessive NHE1 activation in response to ischemic insult leads to cell death (48). Thus, we examined whether Daxx-NHE1 interactions that activate NHE1 sensitize cells to ischemic

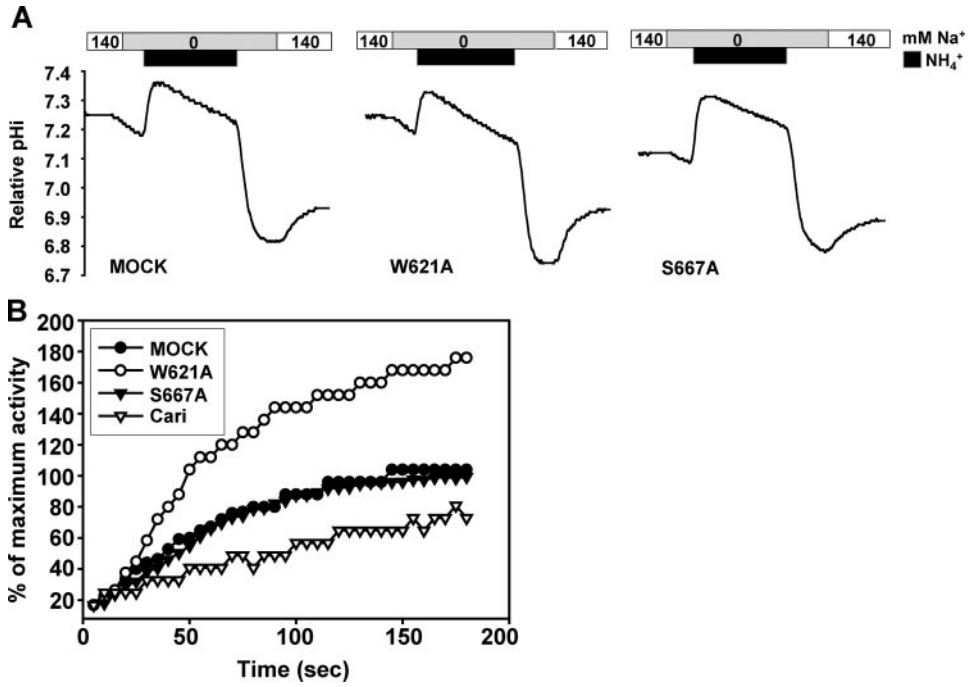

FIGURE 3. **Cytoplasmic Daxx activates NHE1.** *A*, PS120/NHE1 cells were transfected with the mock vector, Daxx(W621A), and Daxx(S667A), respectively. After 24 h of transfection, PS120/NHE1 cells were grown to subconfluence on coverslips. The rate of Na^+/H^+ exchange was measured by determining the rate of intracellular pH (*pHi*) recovery after an acute NH_4Cl-induced acid load using BCECF-AM. *B*, H^+ efflux rates were calculated at various pH_i. Cells were treated with vehicle or 100 nM cariporide before measurement of the Na^+/H^+ exchange rate. *Cari*, cariporide.

insult. CH stimulation killed 25.5 ± 1.4% of cells (Fig. 5*A*, *left panel*). Daxx(W621A) transfection without CH additionally caused significant cell death (19.6 ± 0.1%). Cariporide protected cells from death induced by CH and Daxx(W621A) transfection. This significant protection by cariporide implies that cell death induced by both CH and Daxx(W621A) is attributed mainly to NHE1 ion transporter activity. Daxx(S667A) transfection protected cells from CH-induced death, implying that the cytoplasmic protein is essential for cell death. We further investigated CH-induced death in the NHE-deficient PS120 cells. As shown in Fig. 5*A* (*right panel*), NHE-deficient PS120 cells were significantly resistant to CH. Moreover, transfection of Daxx(W621A) failed to induce cell death. Representative DIC images of cells were obtained using an inverted microscope equipped with DIC optics (Fig. 5*B*). Transfected cells were incubated with cariporide for 30 min before and during CH. Notably, CH- and Daxx(W621A)-induced cell death were inhibited by cariporide. Comparable sensitization and protection in response to CH were observed in relation to the data described for Fig. 5*A*. Changes in cellular morphology were still evident, even in the presence of cariporide. As an additional method of assessing the cell death, cell cytotoxicity was analyzed by lactate dehydrogenase assay following CH (supplemental Fig. S6). Similar sensitization to CH was observed in cells transfected with Daxx(W621A). Also to simulate in another manner, cells were incubated in serum-free, glucose-free Dulbecco's modified Eagle's medium in an anaerobic chamber for the indicated time periods. Similar results were obtained (supplemental Fig. S7). Our data clearly demonstrate that the association of NHE1 and Daxx sensitizes cells to ischemic stress.

DISCUSSION

This study reports molecular interactions between Daxx and NHE1 in response to ischemic damage. Interactions between Daxx and NHE1 lead to the elevation of the NHE1-mediated H^+ transport rate, which eventually increases the intracellular Ca^{2+} level, resulting in cell death. In addition, Daxx inhibits binding of ERM to NHE1 and suppresses activation of the ERM → PI3K → Akt-1 pathway. Using Daxx mutants with defined subcellular localizations, we demonstrate that trafficking of the protein determines two key cellular effects. Cytoplasmic Daxx appears to play a death-promoting role, specifically, excessive stimulation of NHE1 activity, and suppression of the cell survival factor, Akt-1, through competitive binding with ERM to NHE1 protein.

The C-terminal region of Daxx interacts with various proteins, including Fas (1), transforming growth factor-β (22), PML (6), Sentrin (64), Ubc-9 (55), Pax3 (10), Ets-1 (11), and several other transcription factors (65). In addition, the nuclear proteins, X-linked α-thalassaemia mental retardation and signal transducer and activator of transcription 3 (STAT-3), interact with the N terminus of Daxx (9, 66, 67). NHE1 displays more elaborately regulated interaction patterns than the other Daxx-binding partners. First, Daxx-NHE1 interactions are induced in response to cellular energy restriction, and second, nucleocytoplasmic translocation of Daxx is required for these interactions.

A recent study by our group showed that the cytoplasmic presence of Daxx is essential for cell death upon ischemic insult (19). In this investigation, we present preliminary evidence that NHE1 participates in ischemic cell death mediated by cytoplasmic Daxx. In particular, the finding that cariporide protects against cell death induced by cytoplasmic Daxx implies that NHE1 is an essential mediator of this process. The lack of cell death by overexpression of cytoplasmic Daxx in NHE-defective PS120 cells further confirms the pivotal role of NHE1.

We demonstrate that Daxx is a novel positive regulator of NHE1 activity. Previously known activators of NHE1 are mostly kinases, such as mitogen-activated protein kinase ERK1/2 (68), p160ROCK (28), $p90^{RSK}$ (29), and p38 (30), which stimulate NHE1 activity. In view of the importance of NHE1 phosphorylation for its activation, it is possible that interactions with Daxx facilitate phosphorylation by the above kinases. However, further experiments are required to explore this hypothesis.

NHE1-null cells are more sensitive to death than their wild-type counterparts (69, 70), suggesting a positive role for NHE1 in cell survival. Moreover, interactions between NHE1 and

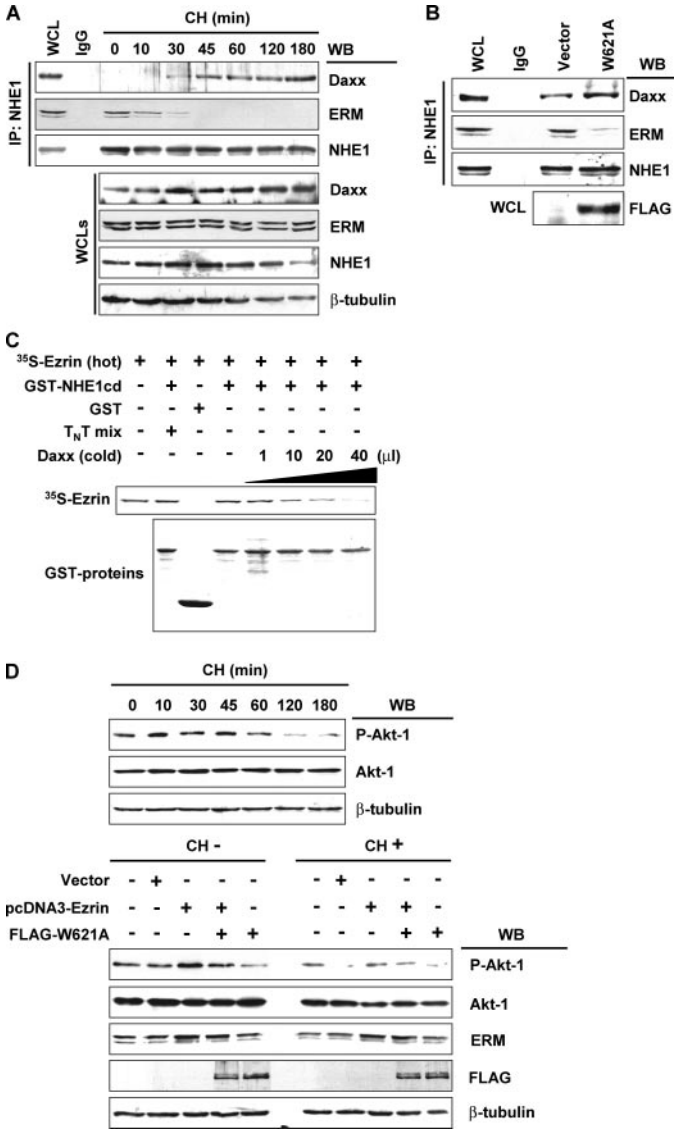

FIGURE 4. **Daxx interferes with NHE1-ERM interactions.** *A*, PS120/NHE1 cells were treated with CH for the indicated periods of time. NHE1 was immunoprecipitated with the anti-NHE1 antibody. Immunoprecipitates were blotted with anti-Daxx, anti-ERM, and anti-NHE1 antibodies. Whole cell lysates (*WCL*) were immunoblotted with the indicated antibodies. *B*, HeLa cells were transfected with the mock vector or FLAG-Daxx(W621A). After 48 h, cell lysates were immunoprecipitated with anti-NHE1 antibody, and immunoblotted with the indicated antibodies. *C*, *in vitro* translated ^{35}S-Ezrin was incubated with immobilized GST, GST-NHE1cd, or *in vitro* translated non-radiolabeled Daxx, as indicated. After washing, resin-bound proteins were analyzed by gel electrophoresis. The *upper panel* depicts the resulting autoradiogram, whereas the *lower panel* shows the Coomassie Blue-stained gel. *D*, PS120/NHE1 cells were exposed to CH for the indicated time periods. Whole cell lysates were harvested at the indicated times and immunoblotted with anti-phospho Akt-1 antibody (*P-Akt-1*). Blots were stripped and reprobed with monoclonal anti-Akt-1 and β-tubulin antibodies (*upper panel*). β-Tubulin was used as an internal standard. PS120/NHE1 cells were transfected with the indicated plasmids. After 48 h, transfected cells were exposed to CH for 2 h. Akt-1 activity was determined by immunoblotting with anti-phospho-Akt-1 antibody (*lower panel*). Blots were stripped and reprobed with the indicated antibodies. The presence of FLAG-Daxx(W621A) in lysates was verified by immunoblotting with the anti-FLAG antibody.

ERM proteins are required for ERM-mediated cell survival signaling (39). Cytoplasmic Daxx prevents NHE1-ERM interactions and suppresses ERM-mediated Akt-1 signaling. Therefore, cytoplasmic Daxx seems to accomplish efficient cell death

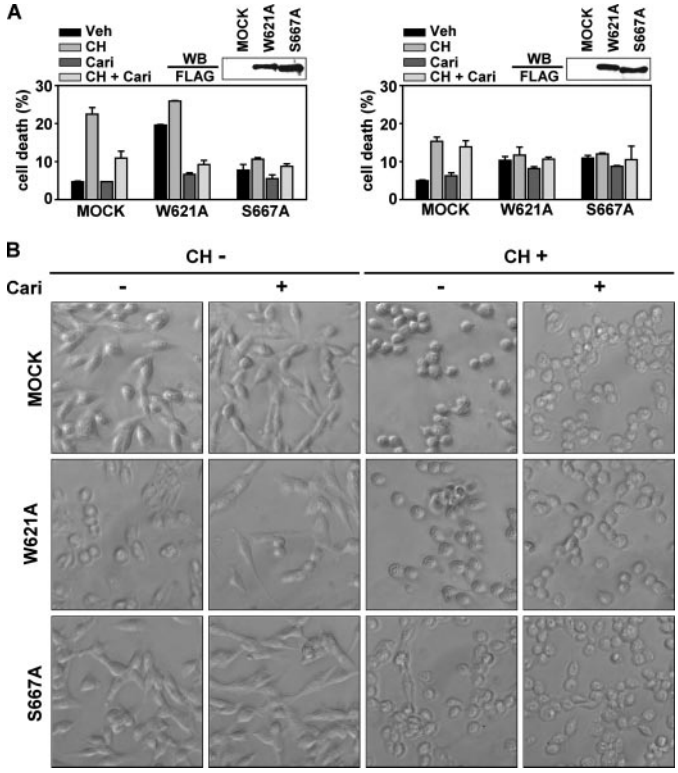

FIGURE 5. **NHE1-Daxx interactions promote CH-induced cell death.** *A*, PS120/NHE1 (*left panel*) and NHE1-deficient PS120 (*right panel*) cells were transfected with the specified plasmids. After 48 h, transfectants were subjected to CH for 2 h, and the pEGFP-C2 vector was used as a co-transfection marker. Transfected cells were incubated with cariporide (10 μM) for 30 min before and during CH. Dead cells were counted using 4′,6-diamidino-2-phenylindole staining. The data in the *bar graph* are mean values ± S.E. ($n = 3$). *B*, transfected cells were prepared as described above. The representative DIC images of cells after 2 h of CH are shown. DIC images were captured using a Carl Zeiss Axiocam MRm camera. Results shown are representatives of three or more experiments performed.

by lowering the cell survival potential in addition to enhancing NHE1 activity. Given the diversity of the Daxx-interacting proteins identified to date, one would expect a complex array of molecules to regulate the effects of Daxx-NHE1 interactions.

Daxx plays a protective role in the nucleus (19). Therefore, complete depletion of Daxx is not beneficial to cells. Molecular agents targeting the nucleocytoplasmic translocation of Daxx should serve as efficient therapeutic tools. Leptomycin B, an inhibitor of exportin-1, which transports Daxx from the nucleus, exhibited a protective potential against ischemic injury (19), supporting this theory. Alternatively, molecules disrupting Daxx-NHE1 interactions may have novel therapeutic purposes, including use as effective anti-ischemic agents.

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Physical Interactions and Functional Coupling between Daxx and Sodium Hydrogen Exchanger 1 in Ischemic Cell Death

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