A Suppressive Role of the Prolyl Isomerase Pin1 in Cellular Apoptosis Mediated by the Death-associated Protein Daxx*

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The death-associated protein Daxx is a multifunctional factor that regulates a variety of cellular processes, including transcription and apoptosis. Several previous reports have indicated that Daxx is induced upon oxidative stress and is then subjected to phosphorylation-based functional modification. However, the precise molecular events underlying these phosphorylation events remain largely unknown. We report in our current study that the peptidyl-prolyl isomerase Pin1 is highly overexpressed in malignant human gliomas and inhibits Daxx-mediated cellular apoptosis. The targeted inhibition of Pin1 by small interferon RNA in A172 glioblastoma cells significantly enhances the cellular apoptotic response, particularly in malignant tumor cells where Pin1 is often overexpressed.

Oncogenesis comprises a complex series of multistep and multifactorial processes that result in uncontrolled cell proliferation, cell transformation, and cell death (1). The resistance to apoptosis in malignant tumor cells is one of the most critical factors that directly contribute to tumor cell proliferation and expansion (2). Furthermore, this apoptotic evasion represents one of the true hallmarks of cancer and appears to be a vital component in chemotherapeutic and radiotherapeutic resistance that characterizes the aggressiveness of human malignant tumors (1).

The antiapoptotic characteristics of tumor cells are often derived from the improper regulation of proapoptotic signaling pathways by various external and internal stimuli (3). One of the pivotal signaling mechanisms that controls cellular apoptotic processes is the phosphorylation of proteins on serine or threonine residues preceding proline (Ser/Thr-Pro) (4, 5). The recent identification and characterization of the peptidyl-prolyl isomerase Pin1, which can recognize these phosphorylated moieties, has led to the elucidation of a number of novel postphosphorylation regulatory mechanisms (4). Pin1 catalyzes the cis-trans isomerization of phosphorylated Ser/Thr-Pro motifs within its specific target substrates (4, 5). Pin1-mediated prolyl isomerization has also now been shown to function in several signaling pathways during tumorigenesis, including Wnt/β-catenin and NF-κB (6, 7). Pin1 is further implicated in many pivotal oncogenic cellular events, such as cell proliferation, angiogenesis, and tumor metastasis (8). However, although some of the roles of Pin1 in several oncogenic signaling pathways have been addressed, there has been no direct evidence reported to date showing that Pin1 can inhibit cellular apoptosis in malignant tumor cells.

The death-associated protein Daxx was originally identified as a Fas-interacting protein that specifically binds to the death domain of Fas and then facilitates Fas-mediated apoptosis independently of FADD (9). Several lines of evidence presented in recent studies have indicated that Daxx plays a crucial role in the cellular apoptotic response induced by UV, oxidative stress, and glucose deprivation, in addition to its function during Fas-mediated apoptosis (10). Daxx has also been reported to localize at the promyelocytic leukemia nuclear bodies in nonapoptotic cells (11). Indeed, nuclear Daxx has been demonstrated to regulate transcription by acting as a transcriptional corepressor via its interaction with several transcription factors (12). Several additional studies have also addressed the potential cyto-

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plasmic versus nuclear roles of Daxx toward the triggering of apoptotic pathways (13). Upon various stimuli, such as serum depletion or oxidative stress, Daxx is phosphorylated and retranslocated from the cytosol to the nucleus via the exportin-mediated nuclear transport system (14). Cytoplasmic Daxx can interact with apoptosis signal-regulating kinase 1 (ASK1)3 and then activate the ASK1/c-Jun-N-terminal kinase (JNK) signaling pathway. In fact, Daxx-depleted cells have been shown to be resistant to cell death pathways induced by both UV irradiation and oxidative stress following impaired ASK1/JNK activation (13). Although the phosphorylation of Daxx has also been shown to regulate its subcellular localization and function following proapoptotic stimuli, it is not known whether its protein stability is regulated by phosphorylation or by other post-translational modifications.

The aim of our present study was to clarify the regulation of Daxx by phosphorylation-dependent prolyl isomerization mediated by Pin1. The importance of both the physical and functional interactions between Daxx and Pin1 during the induction of cell death pathways following exposure to oxidative stress was also investigated, and the involvement of the ASK1/JNK pathway was also evaluated. We find from the results of these analyses that the targeted inhibition of Pin1 in human glioblastoma A172 cells significantly sensitizes these cells to oxidative stress-induced apoptosis, suggesting that Pin1 can protect against the Daxx-mediated apoptotic response. We also find that Pin1 can bind Daxx via its phosphorylated Ser178-Pro motif and facilitate its prompt degradation via the ubiquitin–proteasome pathway. This results in the inhibition of the proapoptotic functions of Daxx. Our present results have thus uncovered a novel molecular mechanism underlying the post-translational regulation of Daxx and demonstrate that Pin1 acts as a putative antiapoptotic molecule in malignant tumor cells.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—Human glioma tissue microarrays (US Biomax, Rockville, MD) were analyzed immunohistochemically using a Pin1 antibody as previously described (15). Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated. After microwave antigen retrieval in sodium citrate buffer, endogenous peroxidase activity was quenched by immersion in 0.3% H2O2. The sections were then treated with anti-Pin1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution for 30 min at room temperature. Biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) was then used as the secondary antibody at a 1:200 dilution for 30 min at room temperature. The sections were subsequently treated using a peroxidase-labeled Vectastain Elite ABC kit (Vector Laboratories), at a 1:200 dilution for 30 min at room temperature. Labeled antigen was visualized via a 3,3′-diaminobenzidine reaction, and each of the sections was counterstained with hematoxylin.

The expression level of Pin1 was evaluated as described previously (15).

Retroviral siRNA Infection—A Pin1-specific siRNA retroviral vector was prepared as previously described (8). Target cell lines were treated with the indicated retrovirus and selected by continuous growth in puromycin (1.0–1.5 μg/ml) for 48 h to isolate stable clones.

siRNA Oligonucleotides—Human Daxx siRNA and scrambled control siRNA oligonucleotides were purchased from Santa Cruz Biotechnology. The final concentration of siRNA oligonucleotides was 200 n mole/liter, and these molecules were introduced into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Cell Culture and Transient Transfections—A172, 293T, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin (100 mg/ml), streptomycin (50 μg/ml). Transient transfections were carried out using either Effectene Transfection Reagent (Qiagen) or HilyMax (DOJINDO, Kumamoto, Japan).

Protein Degradation Assay—Protein degradation assays were performed as described previously (6). Briefly, 293T cells were co-transfected with FLAG-Daxx and either wild-type Pin1 or empty vector, with GFP used as a transfection control. Cycloheximide (50 μg/ml) was added to the medium 24 h after transfection, and the cells were harvested at different time points. Total cell lysates in SDS sample buffer were boiled and then analyzed by immunoblotting with either anti-FLAG (Sigma) or anti-Pin1 (R&D System) or GFP (Invitrogen) antibodies.

GST Pull-down, Immunoprecipitation, and Immunoblotting Analyses—293T cells were lysed with GST pull-down buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 0.5 μg/ml leupeptin, 1.0 μg/ml pepstatin, 10 μM MG132, 10 μM MG115, and 0.2 mM phenylmethylsulfonyl fluoride) and incubated with 30 μl of glutathione-agarose beads containing either GST-Pin1 or GST at 4 °C for 2 h. The precipitated proteins were then washed three times with GST pull-down buffer and subjected to SDS-PAGE (7). For immunoprecipitation, cells were harvested 24 h after transfection and lysed with radioimmuneprecipitation buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 50 mM NaF, 0.5 μg/ml leupeptin, 1.0 μg/ml pepstatin, 10 μM MG132, 10 μM MG115, and 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates were then incubated for 1 h with protein A/G-Sepharose-nonimmunized IgG complexes. Supernatant fractions were recovered and immunoprecipitated with 5 μg of anti-FLAG or anti-Myc antibody and 30 μl of protein A/G-Sepharose. After washing three times with radioimmuneprecipitation buffer, pellets were analyzed on SDS-polyacrylamide gels and subjected to immunoblotting analysis. The antibodies used in this study were obtained from the following sources: mouse monoclonal anti-Daxx, mouse monoclonal anti-Fas (SH-11), and rabbit polyclonal anti-GFP antibodies (MBL International); rabbit polyclonal anti-phospho-ASK1 (Ser83) and cleaved caspase-3 (Asp175) antibodies (Cell Signal-
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In Vitro Kinase Assay—For the measurement of JNK1 activity 
in vitro, A172 cells were lysed in buffer containing 25 mm
HEPES (pH 7.4), 150 mm NaCl, 20 mm β-glycerophosphate, 2
mm EDTA, 2 mm EGTA, 50 mm NaF, 1 mm sodium orthovanadate,
1% Triton X-100, and proteinase inhibitor mixture, as
described previously (18). The lysates were then clarified by
centrifugation and immunoprecipitated with the anti-JNK
antibody C-17 (Santa Cruz Biotechnology) for 2 h. The immune
complexes were recovered with protein A-Sepharose beads and
washed twice with the above lysis buffer and twice with kinase
buffer (25 mm HEPES (pH 7.4), 20 mm MgCl2, 20 mm β-glycerophosphate, 0.5 mm EGTA, 0.5 mm NaF, 0.5 mm sodium orthovanadate)
The immune complexes on the Sepharose beads were used in kinase assays with GST-c-Jun. The reaction
was initiated by adding 30 μl of kinase reaction mixture (kinase buffer plus 5 μCi of [γ-32P]ATP, 20 μM unlabeled ATP, 1 mm
dithiothreitol, and 1 μg of a substrate). After 20 min of incuba-
tion at 30 °C, the reactions were terminated by the addition of
10 μl of 5× SDS-PAGE loading buffer. Samples were resolved by
SDS-PAGE and visualized by autoradiography.

RESULTS

Pin1 Is Highly Expressed in Human Glioma—It has been pre-
viously reported that Pin1 is highly overexpressed in various
human malignancies, including breast and prostate cancers,
and plays a crucial role in oncogenesis (4, 5). Since the rele-
ance of Pin1 in the tumorigenesis of human glioma has not been well
characterized, we investigated the correlation of Pin1 expres-
sion with the malignant properties of human gliomas. To this
end, we first performed immunohistochemical analysis of a
human glioma tissue panel, including normal brain tissue con-
trols (n = 9), low grade astrocytomas (grade 2; n = 24), anaplas-
tic astrocytomas (grade 3; n = 45), and glioblastomas (grade 4;
n = 28), all classified according to World Health Organization
criteria. We found that Pin1 expression was significantly
enhanced in glioma tissues compared with normal brain (Fig.
1A). Interestingly, Pin1 expression was found to be confined to
the nuclei in both normal brain tissue and low grade astrocy-
toma at relatively low expression levels but exhibited enhanced
expression in both the cytoplasm and nuclei of anaplastic astrocy-
toma and glioblastoma (Fig. 1A), as previously found also in other
malignant tumors (15, 19). These immunohistochemical analyses
indicate that the higher expression of Pin1 correlates with a more
highly malignant glioma (Fig. 1B). Our results thus suggest a
potential role for Pin1 in the development of these tumors.

Loss of Pin1 Function Sensitizes Human Glioblastoma Cells to
Oxidative Stress-induced Cell Death—Our immunohisto-
chemical analysis suggested that high levels of Pin1 expression in
human gliomas could contribute to the acquisition of some of the
malignant characteristics of these tumors. It has been
reported that high grade gliomas are resistant to cellular apop-
tosis and that this could be important for tumor cell prolifera-
tion and drug resistance (20). To investigate whether Pin1 con-
tributes to apoptotic resistance in glioma cells, we attempted to
create stable human glioma cell lines in which Pin1 is constitu-
tively suppressed.
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A

B

C

D

E

F

G

H2O2

conti

Pin1i

A172-conti

A172-Pin1i

cell death (%)

hours

0

2

4

0

12

24

conti

Pin1i

H2O2

conti

Pin1i

Apoptotic cells (%)

H2O2

conti

Pin1

Pin1K63A

A172-conti

A172-Pin1i

Pin1

Tubulin

conti

H2O2

Pin1i

Pin1K63A

Apoptotic cells (%)

conti

none

Pin1

Pin1K63A

Pin1i
To this end, we employed a representative human glioblastoma cell line, A172, since these cells have been reported to show antiapoptotic properties against oxidative stress stimuli and anti-tumor drugs (21). The retrovirus-mediated siRNA targeting of Pin1 in A172 cells (A172-Pin1i) was found to cause a marked knockdown of Pin1 expression (<95%), whereas the control siRNA expressing cells (A172-conti) showed Pin1 expression levels that were similar to the noninfected cells (Fig. 2A). We next treated these stable cell lines with either hydrogen peroxide (H₂O₂) or anti-Fas stimulatory antibodies to induce cellular apoptosis. We found that Pin1 depletion by siRNA significantly enhances the rate of cell death caused by both of these stimuli by ~3-fold compared with the control siRNA-expressing cells (Fig. 2, B and C). Consistent with these results, the A172-Pin1i cells exhibit a higher rate of TUNEL staining compared with A172-conti cells when treated with H₂O₂ (Fig. 2, D and E). Furthermore, the forced expression of wild-type Pin1, but not its peptidyl-prolyl isomerase mutant (K63A), which was not subject to knockdown by siRNA, reverted the apoptotic response in cells harboring Pin1 siRNA molecules to control levels (Fig. 2, F and G). These results verify that there is a specific role of endogenous Pin1 in the suppression of H₂O₂- or Fas-induced cellular apoptosis and suggest that the targeted inhibition of Pin1 in glioma cells causes an increased susceptibility to cellular apoptosis induced by oxidative stress.

The Suppression of Pin1 Enhances Daxx Induction and Subsequent ASK1/JNK Activation toward Oxidative Stress-induced Cellular Apoptosis—Our initial analysis indicated that the specific depletion of Pin1 enhances the apoptotic response to oxidative stress in human glioma cells. To delineate the molecular events underlying this phenomenon, we performed immunoblotting analysis to monitor the activity of the intracellular signaling pathways related to oxidative stress-induced cellular apoptosis. Since it has been reported that the activation of the Daxx-ASK1-JNK pathway is a critical event in the induction of cellular apoptosis by H₂O₂ (3), we evaluated the components of this pathway in our current experiments.
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A172-Pin1i cells demonstrate a more prominent induction of the Daxx protein upon H\textsubscript{2}O\textsubscript{2} treatment, compared with control cells (Fig. 3A). Following this enhanced Daxx induction, the phosphorylation of both ASK1 and JNK, indicating activation, was consistently increased in A172-Pin1i cells compared with control cells (Fig. 3A). Furthermore, the levels of cleaved caspase-3 and cleaved PARP, indicating apoptosis, were observed to be significantly higher in Pin1-depleted cells (Fig. 3A), consistent with data from our TUNEL analysis (Fig. 2D). An in vitro kinase assay further demonstrated that JNK activity was significantly increased in A172-Pin1i cells following H\textsubscript{2}O\textsubscript{2} treatment when compared with the control cells (Fig. 3B). These results indicate that the inhibition of Pin1 may sensitize the cells to the apoptotic response induced by H\textsubscript{2}O\textsubscript{2} by augmenting the induction and activation of the Daxx-ASK1-JNK pathway. To confirm this possibility, we transduced Daxx-specific siRNA oligonucleotides to block endogenous Daxx expression as well as treated the cells in parallel experiments with the JNK inhibitor II (SP600125). We found that either Daxx siRNA or SP600125 treatment could significantly reduce the susceptibility of A172-Pin1i cells to H\textsubscript{2}O\textsubscript{2}-induced cellular apoptosis (Fig. 3, C and D). This indicates that the targeted depletion of Pin1 enhances the induction of Daxx, thereby augmenting the apoptotic response via the ASK1/JNK pathway upon oxidative stress.

Pin1 Facilitates the Degradation of Daxx via the Ubiquitin-Proteasome Pathway—Since the depletion of Pin1 was found to enhance the expression of Daxx following oxidative stress, we speculated whether the corollary would be true, such that high levels of Pin1 might in fact inhibit the expression of Daxx. To test this possibility, we initially co-transfected A172 cells with FLAG-tagged Daxx and Pin1 and then examined the expression levels of Daxx. Immunoblotting analysis demonstrated that Pin1 reduces the exogenously transduced FLAG-Daxx levels in a dose-dependent manner (Fig. 4A). Moreover, the expression levels of endogenous Daxx were also found to be reduced in these Pin1-overexpressed cells (Fig. 4B). Interestingly, the mRNA levels of Daxx are not altered upon Pin1 overexpression (data not shown), indicating that Pin1 might affect the protein stability of Daxx.

To address whether either the binding or the catalytic activity of Pin1 is required for the suppression of Daxx, we performed a parallel experiment using either a WW domain (binding domain) mutant (W34A) or PPIase domain (catalytic domain) mutant (K63A) of Pin1. Neither of these mutants fully down-regulate FLAG-Daxx expression (Fig. 4C), indicating that both the WW and PPIase domains are indeed required for this function of Pin1.

**FIGURE 4. Pin1 expression facilitates the degradation of Daxx via the ubiquitin-proteasome pathway.**

A, 293T cells were co-transfected with FLAG-Daxx, GFP, or different amounts of Pin1 expression plasmids. After 24 h, the cells were harvested and subjected to immunoblotting analysis with either anti-FLAG or anti-GFP antibodies. B, 293T cells were transfected with either wild-type Pin1 or empty vector. After 24 h, the cells were subjected to immunoblotting with an anti-Daxx antibody to monitor the endogenous Daxx levels. C, 293T cells were transfected with FLAG-Daxx and co-transfected with either wild-type Pin1 (WT) or its W34A (WW domain) or K63A (PPIase domain) mutants. After 24 h, the cells were subjected to immunoblotting as indicated. D, 293T cells were co-transfected with FLAG-Daxx and GFP and co-transfected with either wild-type Pin1 or empty vector. After 24 h, the cells were treated with H\textsubscript{2}O\textsubscript{2} for 3 h and then subjected to immunoblotting as indicated. E, 293T cells were co-transfected with the indicated vectors, treated with cycloheximide (CHX) after 24 h, and harvested at the indicated time points. This was followed by immunoblotting analysis with either anti-FLAG or anti-GFP antibodies. F, 293T cells were transfected with either Pin1 or control vector subjected to a cycloheximide assay as shown in E. Cells were harvested at the indicated time points followed by immunoblotting analysis. G, 293T cells were subjected to a cycloheximide assay, as shown in E, in the presence or absence of the proteasome inhibitors MG132 and MG115 (10 \mu M each). H, 293T cells were co-transfected with the indicated vectors and treated with cycloheximide (CHX) after 24 h. After a further 12 h, the cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibodies followed by immunoblotting (IB) with anti-FLAG antibodies. Ub\textsubscript{Daxx}, polyubiquitinated. I, 293T cells were co-transfected with the indicated vectors. After 24 h following transfection, cells were treated with the proteasome inhibitor MG132 and MG115 (10 \mu M each) with or without H\textsubscript{2}O\textsubscript{2} for 5 h. The cell lysates were subjected to immunoprecipitation analysis with anti-FLAG antibodies followed by immunoblotting with anti-Myc antibodies. Ub\textsubscript{Daxx}, polyubiquitinated; DMSO, Me\textsubscript{SO}.
Pin1 Interacts with Daxx Phosphorylated on Its Ser<sup>178</sup>-Pro Motif—Our previous results indicated that Pin1 could affect the protein stability of Daxx by mediating the ubiquitination status of this protein. We next examined whether Pin1 could directly interact with Daxx. Immunoprecipitation analysis revealed that this is indeed the case (Fig. 5A). GST pull-down analyses further demonstrated that wild-type Pin1 binds the Daxx protein but that the Pin1 WW domain mutant W34A does not (Fig. 5B). The association between Pin1 and Daxx was also found to be completely abolished by pretreatment of the cell lysates with calf intestine alkaline phosphatase prior to the GST pull-down analysis (Fig. 5C), indicating that Pin1 binds only phosphorylated Daxx. Interestingly, the interaction between Pin1 and Daxx could be observed in both the absence and the presence of H<sub>2</sub>O<sub>2</sub> stimulation (Fig. 5D), indicating that this interaction is independent of the corresponding stress response and that the Pin1 binding motif in Daxx may be constitutively phosphorylated in these cells.

Immunofluorescence analysis further demonstrated that Pin1 co-localizes with Daxx in intranuclear aggregates corresponding to promyelocytic leukemia bodies in the absence of H<sub>2</sub>O<sub>2</sub> stimulation, as reported previously (11) (Fig. 5D). Upon H<sub>2</sub>O<sub>2</sub> stimulation, certain subsets of both Daxx and Pin1 were found to translocate diffusely into the cytoplasm, although the majority of these proteins were still retained in the nucleus and colocalized together in nuclear bodies (Fig. 5D).

To identify the specific Pin1 binding site in the Daxx protein, we created several Daxx deletion mutants and performed GST pull-down analysis. These experiments revealed that an N-terminal Daxx deletion mutant (Δ1–36) could still bind Pin1 but also that an extended N-terminal deletion mutant (Δ1–183) failed to do so (Fig. 6A). These data indicate that Pin1 binds to Daxx in the region between amino acids 36 and 183.

Previous reports have indicated that Pin1 can bind only phosphorylated Ser/Thr-Pro motifs (4, 5). Since there is only a single Ser/Thr-Pro motif (Ser<sup>178</sup>-Pro) between residues 36 and 183 in the Daxx protein, we created a Daxx site-directed mutant at this site by substituting the serine 178 with alanine (S178A). Moreover, we created an additional Daxx mutant by substituting serine 668 with alanine (S668A), since this site has been shown to be phosphorylated (14). Both GST pull-down and immunoprecipitation analyses subsequently revealed that Pin1 binds both wild-type and the S668A mutant Daxx proteins but not the S178A mutant (Fig. 6, B and C). These results confirm that Pin1 indeed binds the phosphorylated Ser<sup>178</sup>-Pro motif of Daxx.
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The Daxx-S178A Mutant Is Refractory to Pin1-mediated Degradation and Shows Strong Proapoptotic Properties—To further examine the functional interactions between Pin1 and Daxx, we initially investigated the nature of the S178A mutant in terms of its protein stability. Cycloheximide analysis revealed that the S178A Daxx mutant is resistant to degradation following the co-transfection of Pin1 (Fig. 7A). Consistent with this result, this S178A mutant also shows lower levels of ubiquitination compared with wild-type Daxx upon Pin1 co-transfection (Fig. 7B). A reciprocal immunoprecipitation analysis further revealed that the S178A mutant was refractory to be polyubiquitinated following Pin1 overexpression as compared with wild-type Daxx (Fig. 7C). These results together confirm that the direct interaction between Pin1 and Daxx via the Ser<sup>178</sup>-Pro motif augments the ubiquitination of Daxx and thereby enhances its degradation by the proteasome.

We next examined the proapoptotic properties of the S178A Daxx mutant in the absence or presence of exogenous Pin1. A previous report has indicated that the co-transfection of Daxx and its downstream target ASK1 initiates apoptosis in HeLa cells (22). We therefore co-transfected HeLa cells with ASK1 and either FLAG-Daxx or S178A Daxx in the presence or absence of Pin1. As shown in Fig. 7, D and E, the co-transfection of ASK1 with either wild-type or S178A mutant Daxx results in cellular apoptosis. However, the S178A mutant shows stronger proapoptotic effects compared with wild type Daxx. Furthermore, when co-transfected with Pin1, the S178A mutant still retains its potent ability to induce cellular apoptosis, which is in contrast to wild-type Daxx, which fails to induce apoptosis in the presence of high levels of Pin1, as revealed by either immunostaining for cleaved caspase-3 or Hoechst 33258 staining for apoptotic nuclei (Fig. 7, D and E). These results together indicate that the proapoptotic properties of the S178A mutant are refractory to the anti-apoptotic function of Pin1.

Reverse Correlation between Pin1 and Daxx Expression in Human Glioblastoma Tissues—To further examine the pathological role of Pin1 in the degradation of Daxx, we determined the expression levels of the Daxx protein in the 28 grade 4 human glioblastoma tissues that we analyzed earlier for Pin1 expression by immunohistochemical staining. As shown in Fig. 1, Pin1 is expressed to various degrees in human glioblastoma tissues. Consistent with our molecular data, we found that Daxx staining was predominantly absent in glioma tissues containing high levels of Pin1 and that Daxx accumulation in the nucleus was evident in cases containing relatively low expression levels of Pin1 (Fig. 8A). Among the 28 grade 4 human glioblastoma tissues that we examined, there was also a significant reverse correlation between Pin1 expression and the immunoreactivity of Daxx, as determined by the Spearman rank correlation test (p < 0.01) (Fig. 8B). These results further support the notion that Pin1 is important for the regulation of Daxx expression in vivo and strengthen the significance of Pin1 overexpression in the negative regulation of Daxx in malignant human glioma.

DISCUSSION

In our current study, we report that the peptidyl-prolyl isomerase Pin1 associates with phosphorylated Daxx and enhances its degradation, resulting in the prevention of oxidative stress-induced cellular apoptosis. We find that 1) Pin is highly overexpressed in human gliomas, and its expression levels parallel the malignant properties of the glioma cells; 2) Pin1-depleted A172 glioblastoma cells are highly susceptible to cellular apoptosis induced by either hydrogen peroxide or stimulatory Fas antibodies; 3) Pin1 inhibition enhances the induction of Daxx and the activation of the ASK1/JNK apoptotic pathway; 4) Pin1 overexpression causes the rapid degradation of the Daxx protein via the ubiquitin-proteasome pathway; 5) Pin1 interacts with Daxx via its phosphorylated Ser<sup>178</sup>-Pro...
motif, and the Daxx-S178A mutant is refractory to both Pin1-mediated degradation and shows strong pro-apoptotic properties in the presence of Pin1.

Cancer cells often exhibit several types of malignant behavior, including a self-sufficiency in terms of growth signals, insensitivity to growth-inhibitory signals, and the ability to evade programmed cell death (1). One of the current major issues in the clinical treatment of human glioma is the resistance of many of these tumors to chemotherapy (2). In our current report, however, we show that Pin1 inhibition increases the sensitivity of glioma cells to both H2O2 and Fas-mediated apoptosis and that this is accompanied by the increased expression of Daxx. In contrast, Pin1 overexpression significantly suppresses the expression of Daxx, thereby enhancing its degradation via the ubiquitin-proteasome pathway. These data suggest that aberrantly high levels of Pin1 in tumor cells can contribute to a blockade of proapoptotic pathways and promote the inappropriate survival of tumor cells. The inhibition of Pin1 may therefore be an effective strategy for the future treatment of glioma.

The involvement of Pin1 in cellular apoptosis has been addressed previously. First, Pin1 has been shown to interact with both p53 and p73, thereby affecting their stability. This modifies both the cell cycle-regulatory mechanisms and apoptotic pathways induced by genotoxic stress stimuli (23–25). Second, Pin1 also binds the antiapoptotic protein Bcl-2 during mitosis at a proline-rich loop region, thereby blocking its cytoprotective and ion channel-forming activities (26). Pin1 has also been reported to interact with the BH3-only protein BIMEL, thus inducing apoptosis in neurons (27). These results indicate multiple effects of Pin1 on cellular apoptosis in different tissues or cell types via its interaction with specific substrate proteins. However, given that the Daxx-ASK1-JNK pathway plays a crucial role in cellular apoptosis by various proapoptotic stimuli and that this signaling pathway is often deregulated in many human tumors (10), our current study uncovers an important molecular mechanism by which malignant tumor cells with aberrantly high Pin1 levels could evade Daxx-mediated apoptosis. This in turn could directly contribute to tumor expansion and resistance to anticancer therapies.

It has been suggested that the subcellular localization of Daxx might determine its activity and function toward the induction of proapoptotic signaling, and this is critically regulated by multiple related factors. Several lines of evidence have now indi-
The regulation of Daxx nuclear export appears to be dependent on its phosphorylation on Ser668, potentially mediated by the HIPK1 kinase (14). This in turn enhances the interaction of Daxx with the nuclear exporter CRM1, which controls its phosphorylation-dependent translocation to the cytoplasm (14). In summary, we demonstrate herein that Pin1 is a negative regulator of Daxx and demonstrate a novel regulatory mechanism of Daxx involving phosphorylation-dependent prolyl isomerization. The targeted inhibition of Pin1 could therefore be a valid therapeutic strategy to induce cellular apoptosis in malignant tumors. This includes gliomas in which aberrantly high Pin1 expression is often observed.

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FIGURE 8. Reverse correlation between Pin1 and Daxx expression in human glioblastoma tissues. A, human glioblastoma tissues were stained with anti-Daxx or anti-Pin1 antibody followed by staining with dianinobenzene (DAB; brown). Left, a representative tumor showing strong staining for Pin1 and negative Daxx staining; right, a representative low Pin1-stained glioma with an accumulation of Daxx in the nucleus. Nuclei were stained weakly with hematoxylin. Inset, a focal magnification of Daxx staining in nuclear bodies. B, a summary of immunohistochemical analysis of a 28-human glioblastoma tissue panel is shown. The levels of Pin1 and Daxx expression were determined, and a significant reverse correlation was confirmed using a Spearman rank test (p < 0.01).
Pin1 Facilitates the Degradation of Daxx

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