Mitochondrial p53 Contributes to Reovirus-Induced Neuronal Apoptosis and Central Nervous System Injury in a Mouse Model of Viral Encephalitis

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ABSTRACT

The tumor suppressor p53 plays a critical part in determining cell fate both as a regulator of the transcription of several proapoptotic genes and through its binding interactions with Bcl-2 family proteins at mitochondria. We now demonstrate that p53 protein levels are increased in infected brains during reovirus encephalitis. This increase occurs in the cytoplasm of reovirus-infected neurons and is associated with the activation of caspase 3. Increased levels of p53 in reovirus-infected brains are not associated with increased expression levels of p53 mRNA, suggesting that p53 regulation occurs at the protein level. Increased levels of p53 are also not associated with the increased expression levels of p53-regulated, proapoptotic genes. In contrast, up-regulated p53 accumulates in mitochondria. Previous reports demonstrated that the binding of p53 to Bak at mitochondria causes Bak activation and results in apoptosis. We now show that Bak is activated and that activated Bak is bound to p53 during reovirus encephalitis. In addition, survival is enhanced in reovirus-infected Bak^{-/-} mice compared to controls, demonstrating a role for Bak in reovirus pathogenesis. Inhibition of the mitochondrial translocation of p53 with pifithrin μ prevents the formation of p53/Bak complexes following reovirus infection of *ex vivo* brain slice cultures and results in decreased apoptosis and tissue injury. These results suggest that the mitochondrial localization of p53 regulates reovirus-induced pathogenesis in the central nervous system (CNS) through its interactions with Bak.

IMPORTANCE

There are virtually no specific treatments of proven efficacy for virus-induced neuroinvasive diseases. A better understanding of the pathogenesis of virus-induced CNS injury is crucial for the rational development of novel therapies. Our studies demonstrate that p53 is activated in the brain following reovirus infection and may provide a therapeutic target for virus-induced CNS disease.

Viral encephalitis causes morbidity and mortality throughout the world (1, 2). New therapies, including those targeting specific mechanisms of virus-induced central nervous system (CNS) injury, are urgently needed. Intracerebral injection of neonatal mice with type 3 (T3) strains of reovirus is a well-characterized experimental model for studying viral pathogenesis within the CNS. Reovirus specifically targets neurons within the hippocampus, thalamus, and cortex, causing lethal encephalitis. We and others have previously shown that apoptosis of infected neurons is an important pathogenic mechanism during reovirus encephalitis and that reovirus-induced neuronal apoptosis involves the activation of both extrinsically (death receptor) and intrinsically (mitochondrial) mediated pathways, leading to the activation of cellular caspases (3–7).

The tumor suppressor p53 plays a critical role in cell death signaling following a variety of insults (8, 9), including infection of BHK-21 cells with avian reovirus (10) and during reovirus oncolysis (11). Increased levels of p53 can promote cell death by modulating the expression/activity of Bcl-2 family proteins, which regulate mitochondrion-mediated cell death pathways. The transcriptional activity of p53 (12) can promote intrinsic apoptosis by upregulating Bax and PUMA (p53-upregulated modulator of apoptosis). Alternatively, the mitochondrial accumulation of p53 can contribute to intrinsic cell death signaling by activating Bax and Bak (13, 14). Activated Bak and Bax form pores in the outer mitochondrial membrane, resulting in the release of proapoptotic

proteins, including cytochrome *c* (cyt *c*) and second mitochondrial activator of caspases (SMAC) (15, 16). We have previously shown that Bax is activated and present at mitochondria during reovirus encephalitis (6). In addition, reovirus CNS infection of mice deficient in Bax expression results in reduced cyt *c* and SMAC release, decreased neuronal apoptosis and CNS injury, and increased survival compared to infection of wild-type (WT) controls (6). We now show that the mitochondrial protein Bak is also activated in the brain following reovirus infection and contributes to virus-induced CNS disease. Bak activation is associated with the upregulation of p53 in reovirus-infected brains and translocation of p53 to the mitochondria, where it is present in a complex with activated Bak. These results suggest that p53 contributes to reovirus pathogenesis in the CNS via its interactions with Bak.

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MATERIALS AND METHODS

Viral stocks. Reoviruses serotype 3 strain Dearing (T3D) and serotype 3 strain Abney (T3A) are laboratory stocks derived via plaque purification and double passage in L929 (ATCC CCL1) cells. Virus stocks are further purified via high-speed cesium chloride density gradient centrifugation.

In vivo studies. Swiss Webster (SW) outbred mice were obtained from Harlan Laboratories (Indianapolis, IN). Neonatal Bak-deficient (Bak^{-/-}) mice, strain B6.129-Bak1^{tm1Thsn/J} mice, and WT mice (strain B6.129X1-Bax^{tm1Sjk/J}) were purchased from the Jackson Laboratory (Bar Harbor, ME) (17). Strain B6.129X1-Bax^{tm1Sjk/J} was used as a control since these mice are more closely related to Bak^{-/-} mice than pure C57BL/6J mice. Two-day-old mice were inoculated intracranially (i.c.) with T3D (100 PFU or as indicated) diluted in a 10- μ l volume as described previously (6). Mock-infected mice were injected i.c. with an equal volume of phosphate-buffered saline (PBS). For survival analysis, mice were monitored up to 21 days following infection. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

Western blot analysis. Immediately upon harvest, whole brains were triturated in lysis buffer in the presence of a phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Lysates were cleared, electrophoresed through 10% polyacrylamide gels at a constant 120 V, and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Pittsburgh, PA). Immunoblotting was performed with anti-p53 (catalog number FL-393; Santa Cruz Biotechnology, Dallas, TX), anti-Bak (catalog number 12105; Cell Signaling, Danvers, CA), anti-Bak, N terminus (anti-Bak-NT) (catalog number ABC12; Millipore, Billerica, MA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (catalog number 2118; Cell Signaling, Danvers, CA), and anti- β -actin (Calbiochem, Sunnyvale, CA) antibodies. Following washes and 1 h of incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), images were obtained on a FluorochemQ MultiImage III imaging workstation (Cell Biosciences, Santa Clara, CA), and densitometry quantification was performed with Alphaview v3.0 software (18).

Histological studies and immunocytochemistry staining. Freshly harvested brain tissue was immersed in 10% buffered formalin for no less than 12 h, embedded in paraffin, and serially sectioned into 6- μ m-thick sections, before being mounted onto slides. Sections were desiccated at 50°C for 15 min and then rehydrated in PBS over 30 min. Tissue was subjected to antigen retrieval (antigen-unmasking solution; Vector Laboratories, Burlingame, CA) and permeabilization/blocking (5% fetal bovine serum [FBS] and 5% normal goat serum in 0.3% Triton-PBS) prior to incubation with the following primary antibodies: monoclonal reovirus σ 3 antibody (4F2) (1:100), rabbit polyclonal reovirus antibody (1:200), mouse anti-MTCO2 (cytochrome *c* oxidase subunit II, mitochondrion marker) antibody (1:200) (catalog number ab91317; Abcam, Cambridge, MA), mouse anti-neuronal nuclear antibody (NeuN) (1:100) (catalog number ABN78; Millipore, Billerica, MA), chicken anti-neuronal marker microtubule-associated protein 2 (MAP2) antibody (1:200) (catalog number ab5392; Abcam, Cambridge, MA), rabbit anti-cleaved caspase 3 antibody (1:50) (catalog number 9661; Cell Signaling, Danvers, CA), and/or rabbit anti-p53 antibody (1:100) (catalog number FL-393; Cell Signaling, Dallas, TX). Sections were then incubated with the following secondary antibodies: Alexa Fluor 488-conjugated IgG, Alexa Fluor 568-conjugated IgG, Alexa Fluor 647-conjugated IgG (1:200; Invitrogen, Carlsbad, CA), and/or Cy3-conjugated AffiniPure IgG (1:300; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with Hoechst stain (10 μ g/ml; Immunochemistry Technology, Bloomington, MN) for immunofluorescence imaging. Sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and slides were imaged either on a Marianas fluorescence imaging workstation (Intelligent Imaging Innovation Inc., Denver, CO) based on a Zeiss 300M inverted microscope or on an Olympus FV1000 confocal microscope with Olympus FluoView software (Olympus, Center Valley, PA) (18).

Mitochondrion isolation. Mitochondria were isolated from mouse brain with a mitochondrion isolation kit for tissue with a reagent-based method according to the manufacturer's instructions (catalog number 89801; Thermo, Rockford, IL). Briefly, brain homogeneous suspensions were prepared with a prechilled Dounce homogenizer. Brain homogeneous suspensions were spun at 1,000 $\times$ g for 5 min, and the pellet was suspended in 800 μ l of a bovine serum albumin (BSA)-reagent A solution and incubated for 2 min before the addition of 10 μ l isolation reagent B. After incubation for 5 min with intermittent vortexing, 800 μ l of reagent C was added. The resultant cell lysate was centrifuged at 700 $\times$ g for 10 min, and the supernatant was transferred to a new tube and spun at 3,000 $\times$ g for 15 min. The supernatant (cytosolic fraction) was collected for analysis. After washing with mitochondrion isolation reagent C, the mitochondrial pellet was lysed with 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in Tris-buffered saline containing protease inhibitors. All procedures were performed at 4°C.

Coimmunoprecipitation. Coimmunoprecipitations were performed by using a Pierce Classic immunoprecipitation (IP) kit according to the manufacturer's protocol (catalog number 26146; Thermo, Rockford, IL). Briefly, 600- μ g mitochondrial fractions prepared from mouse brains were precleared by using control agarose resin. The precleared lysate was immunoprecipitated with 4 μ g anti-Bak-NT (catalog number ABC12; Millipore, Billerica, MA), anti-p53 (catalog number ab26; Abcam, Cambridge, MA), or isotype antibodies and incubated at 4°C for overnight. The immune complex was captured with Pierce protein A/G-agarose. After extensive washings with IP lysis/washing buffer, the immune complex was eluted with 2 $\times$ sample buffer provided in the kit, and the precipitated proteins were loaded onto Tris-glycine gels, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies as indicated. For the input control, 50 μ g of the lysate prior to immunoprecipitation was also loaded onto Tris-glycine gels. Western blotting was performed as described above.

Organotypic brain slice culture studies. Brain slice cultures (BSCs) were prepared from 2- to 3-day-old mice and infected with 10⁶ PFU T3A reovirus as previously described (18, 19). Briefly, four 400- μ m coronal sections of cerebrum were made from a single animal by using a vibrating-blade microtome (catalog number VT1000S; Leica, Bannockburn, IL). Slices were maintained on a semiporous membrane insert (catalog number PICMORG50; Millipore, Billerica, MA) and in a 35-mm tissue culture well containing 1.1 ml serum-containing medium. Immediately after plating, slices were infected by the dropwise addition of 10⁶ PFU T3A (diluted in 20 μ l PBS) or PBS only to each slice. Medium was refreshed with 5% FBS-containing medium $\sim$ 12 h after slicing, and subsequent medium changes were made with serum-free medium every 2 days thereafter. For inhibitor studies, pifithrin μ was added to the medium at the time of virus infection and was maintained every 2 days during subsequent medium changes.

Lactate dehydrogenase assay. For measurement of injury in organotypic slices, BSC medium was collected at various times postinfection (p.i.), and triplicate 10- μ l samples were analyzed with LDH-Cytotoxicity Assay kit II (BioVision, Mountain View, CA, USA), in which fresh medium was used as a blanking control and purified lactate dehydrogenase (LDH) was used as a positive control. LDH activity was quantified on a microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA) as a function of the optical density measured at 450 nm with wavelength correction at 650 nm, as described previously (18, 19).

Caspase 3 fluorometric assay. Caspase 3 activity was determined by utilizing a fluorogenic assay (catalog number BF1100; R&D Systems, Minneapolis, MN). The reaction products were measured on a Cytofluor series 4000 spectrofluorometer (Applied Biosystems, Carlsbad, CA) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Statistical analysis. All statistical analyses were performed by using Prism software (GraphPad Software Inc., San Diego, CA). Statistical comparisons of mean measurements between two groups (e.g., mock versus infected) were made by using a two-tailed, unpaired *t* test with Welch

FIG 1 p53 protein levels are increased following reovirus infection of the brain. Two-day-old SW mice were inoculated i.c. with 100 PFU reovirus T3D or an equal volume of PBS (mock). Brains were harvested 9 days following infection. (A) Western blotting was performed on whole-cell lysates by using antibodies directed against p53, reovirus (Reo) $\sigma 3$ (4F2 antibody), and cleaved caspase 3 (CC3). Antiactin antibodies were used to control for protein loading. The blots demonstrate increased levels of p53 following reovirus infection of the CNS. (B) Densitometric analysis of 3 blots showing the average intensity (arbitrary units) of bands from 3 mock- and reovirus-infected mice and demonstrating a 2.8-fold increase in the p53 level in reovirus-infected compared to mock-infected brains. Error bars indicate standard deviations. (C and D) Fluorescent immunohistochemical staining reveals that following reovirus infection, p53 is upregulated in the hippocampus (hippo) and is found largely within cells that are positively labeled for reovirus $\sigma 3$ (4F2). Examples of cells staining positive for reovirus antigen and p53 are highlighted with arrows. Immunohistochemical images are representative fields from at least 3 individual reovirus-infected and mock-infected brains. The original magnifications are indicated. Bars, 20 μ m.

correction. A log rank test was used to compare Kaplan-Meier survival curves for different groups of infected mice. A *P* value of <0.05 was considered significant.

RESULTS

Levels of p53 are increased in the brain following reovirus infection. To investigate the role of p53 during reovirus encephalitis, we inoculated 2-day-old Swiss Webster (SW) mice with 100 PFU of reovirus (T3 Dearing [T3D]) by i.c. injection (6). Brains were harvested at 9 days p.i., when mice were showing clinical signs of disease, and whole-cell lysates were examined for p53 levels by Western blotting (Fig. 1A). Densitometric quantification showed that infection of the brain with reovirus resulted in a 2.8-fold increase in the level of p53 protein ($P = 0.02$) compared to mock-infected controls (Fig. 1B). Upregulated p53 following reovirus infection was associated with apoptosis, as demonstrated by increased levels of cleaved/activated caspase 3 in reovirus-infected brains compared to mock-infected controls (Fig. 1A).

Previous studies by ourselves and others have demonstrated that reovirus specifically targets the hippocampus (CA1 to CA3 regions), cortex, and thalamus (20). Immunohistochemistry performed on brain slices from mock- and reovirus-infected mice

(see above) demonstrated that p53 is upregulated in the hippocampus (Fig. 1C), cortex, and thalamus (not shown) during reovirus encephalitis and occurs predominantly in reovirus-infected cells (Fig. 1D). Colabeling with the neuronal marker MAP2 identified these p53/reovirus-expressing cells as neurons (Fig. 2A). Occasional neurons staining positive for p53 but not reovirus antigen could sometimes be seen (Fig. 2A), which may represent a bystander effect or may simply be related to the timing of infection. Reovirus-infected neurons were also shown to undergo apoptosis, as demonstrated by the activation/cleavage of caspase 3 (Fig. 2B). Activated caspase 3 could sometimes be seen in cells that did not show reovirus and/or MAP2 staining (Fig. 2B). It is unclear whether this is indicative of nonneuronal cell apoptosis or a loss of MAP2 during neuronal death.

p53 translocates to the mitochondria in reovirus-infected brains. Increased levels of p53 have been shown to promote cell death by transcriptionally activating proapoptotic genes, including Bax and PUMA. Our prior microarray studies indicated that Bax and PUMA are not upregulated in the brain throughout the course of reovirus infection (21), suggesting that the transcriptional activity of p53 does not contribute to pathology during

FIG 2 p53 is upregulated in reovirus-infected neurons. Two-day-old Swiss Webster mice were inoculated i.c. with 100 PFU T3D (Reo) or an equal volume of PBS (mock). Brains were harvested at 9 days p.i. (A) Immunohistochemistry using antibodies directed against reovirus ($\sigma 3$), p53, and the neuronal marker MAP2 and Hoechst staining shows that p53 is upregulated in neurons, the majority of which also stain positive for reovirus antigen (large arrows). Occasional cells staining positive for p53 and NeuN but not reovirus were occasionally seen (small arrows). (B) Immunohistochemistry using antibodies directed against reovirus, cleaved caspase 3 (CC3), and MAP2 and Hoechst staining shows that reovirus-infected neurons undergo apoptosis (arrows). Bars, 20 μ m.

reovirus encephalitis. The cytoplasmic location of p53 (Fig. 1 and 2) is also consistent with a non-transcriptionally mediated mechanism of cellular death. To investigate whether p53 translocates to the mitochondria following reovirus infection of the brain, mice were infected as described above, and Western blot analysis was performed on mitochondrial and cytosolic fractions prepared from reovirus- and mock-infected brains. Infection of the brain with reovirus resulted in a 4.5-fold increase in the level of mitochondrial p53 ($P = 0.001$) compared to mock-infected controls (Fig. 3A and B). In contrast, levels of cytosolic p53 were not increased following reovirus infection. These results demonstrate that p53 translocates to the mitochondria during viral encephalitis.

Bak is activated in the brain during reovirus encephalitis. Translocation of p53 to the mitochondria can trigger cell death by binding to and activating proapoptotic Bcl-2 family proteins, including Bax and Bak. We have previously shown that Bax is activated in the brain following reovirus infection (6). To determine whether Bak is activated in the brain following reovirus infection, mice were infected with reovirus as described above. Activated Bak was then immunoprecipitated from cell lysates by using antibodies directed against conformationally activated Bak (Bak-NT).

Western blotting, using anti-full-length Bak antibodies, was then used to show that levels of activated Bak are increased during reovirus encephalitis (Fig. 4A). Immunohistochemical analysis showed that activated Bak colocalizes with the mitochondrial marker TOM40 (translocase of outer mitochondrial membrane 40) in reovirus-infected cells (Fig. 4B). These results demonstrate that Bak is activated in the brain following reovirus infection and confirm that activated Bak is present at the mitochondria. Increased survival in reovirus-infected Bak-deficient mice (60% survival), compared to WT controls (10% survival), then demonstrated that Bak plays a role in reovirus pathogenesis in the CNS (Fig. 4C).

Immunoprecipitation experiments using bait antibodies generated against either Bak-NT or p53 were then used to demonstrate the formation of p53 complexes with activated Bak in reovirus-infected brains (Fig. 5). No p53/activated Bak complexes were immunoprecipitated from infected lysates by using isotype control antibodies or from mock-infected lysates.

Pifithrin μ inhibits reovirus-induced apoptosis and injury in BSCs. Pifithrin μ is an inhibitor of p53-mediated apoptosis that prevents p53 binding to mitochondria by reducing its affinity for the antiapoptotic mitochondrial proteins Bcl-2 and Bcl-XL, while

FIG 3 p53 translocates to mitochondria in reovirus-infected brains. Two-day-old Swiss Webster mice were inoculated i.c. with 100 PFU T3D (Reo) or an equal volume of PBS (mock). Brains were harvested at 9 days p.i. (A) Mitochondrial and cytosolic fractions were prepared from whole-cell lysates and were analyzed by Western blot analysis using antibodies against p53, the mitochondrial marker (voltage-dependent anion channel [VDAC]), and GAPDH. (B) Densitometric analysis showing a 4.5-fold increase in the level of p53 in the mitochondrial fraction of reovirus-infected brains compared to mock-infected controls. The graph shows the amounts of p53 (normalized to VDAC) from at least 3 individual animals. Error bars represent standard deviations.

having no effect on the transactivational or cell cycle checkpoint control function of p53 (22). We used BSCs that were treated with 60 μ M pifithrin μ to demonstrate that mitochondrial translocation of p53 contributes to reovirus CNS pathogenesis. For these experiments, BSCs were prepared from 2-day-old SW mice and were infected with 1×10^6 PFU reovirus (T3A)/slice as previously

described (19). We utilized T3A in BSCs since it causes more consistent pathology than T3D. Pifithrin μ was added to the medium at the time of virus infection and was maintained every 2 days during subsequent medium changes. Nine days following infection, slices were harvested, and caspase 3 activity was measured by using fluorogenic activity assays. Reaction products, measured on

FIG 4 Bak is activated in the brain following reovirus infection and contributes to virus pathogenesis. Two-day-old Swiss Webster mice were inoculated i.c. with 100 PFU T3D (Reo) or an equal volume of PBS (mock). Brains were harvested at 9 days p.i. (A) Whole-cell extracts from individual mice were incubated with anti-Bak-NT or isotype control (iso) antibodies, and the immunoprecipitated lysate was immunoblotted and probed with anti-Bak (full-length) antibody. Input controls showing equal amounts of Bak in the input are also shown. (B) Fluorescent immunohistochemical staining demonstrating that Bak-NT (green) colocalizes with the mitochondrial marker TOM40 (red) in reovirus-infected cells following reovirus infection (arrows). The image shows a representative field from the hippocampus. (C) Two-day-old WT ($n = 17$) and Bak^{-/-} ($n = 12$) mice were injected with 20 PFU T3D reovirus and were monitored for survival for 21 days p.i. The graph shows a significant increase in survival in Bak^{-/-} compared to WT mice ($P < 0.001$ by a log rank test).

FIG 5 Activated Bak binds to p53 in reovirus-infected brains. Two-day-old Swiss Webster mice were inoculated i.c. with 100 PFU T3D (Reo) or an equal volume of PBS (mock). Brains were harvested at 9 days p.i. Bak (A) or p53 (B) was immunoprecipitated from whole-cell lysates by using antibodies directed against p53 (A) or Bak-NT (B) or isotype controls (iso). Precipitated proteins were immunoblotted.

a Cytofluor series 4000 spectrofluorometer, showed that pifithrin μ significantly reduced reovirus-induced activation of caspase 3 in BSCs (Fig. 6A). Lactate dehydrogenase assays, performed on media from mock- and reovirus-infected BSCs, demonstrated that pifithrin μ also significantly reduced reovirus-induced tissue injury (Fig. 6B). These results show that the mitochondrial localization of p53 is important for reovirus pathogenesis in the CNS. Western blotting of immunoprecipitated proteins from cellular lysates prepared from reovirus- and mock-infected BSCs demonstrated that pifithrin μ inhibits the formation of p53/activated Bak complexes following reovirus infection, identifying a new Bcl-2 family binding interaction of p53 and suggesting that the interaction of p53 and Bak also contributes to pathogenesis during reovirus encephalitis (Fig. 6C).

DISCUSSION

In this paper, we show that p53 is upregulated in virus-infected neurons during reovirus encephalitis. p53 mRNA is not upregulated in reovirus-infected brains, suggesting that virus infection manipulates p53 expression at the protein level. The most likely explanation for the increased p53 protein levels, in the absence of increased p53 mRNA levels, is a decrease in p53 turnover, a phenomenon known as p53 “stabilization.” This process involves the inhibition of Mdm2, which normally regulates the level of p53 by transporting it to the cytoplasm for proteosomal degradation. Previous studies demonstrated that West Nile virus (WNV)-induced apoptosis *in vitro* requires p53 stabilization (23, 24). Other possibilities resulting in higher protein levels without changes in mRNA levels include increased translation of p53 RNA.

Upregulated p53 translocates to the mitochondria in reovirus-infected neurons, where it interacts with mitochondrial proteins, including the proapoptotic Bcl-2 family protein Bak. p53 has been shown to bind and activate Bak in other systems, including low-temperature cell stress (25), DNA strand breaks induced by camptothecin (CPT) (14), and chemotherapeutic drug administration (26).

Bak was previously investigated in other models of encephalitis and noninfectious causes of neuronal apoptosis, and its

role is still a matter of controversy. Bak upregulation occurs in the CNS during rabies virus infection (27), and Bak is antiapoptotic or proapoptotic depending upon the age of the mice and the neuronal subtype during Sindbis virus encephalitis (28). Bak is important for neuronal apoptosis induced by oxidative stress or DNA-damaging agents in primary neurons, cochlear cells, and neuroblastoma cells (29). Bak may also regulate neurotransmitter signaling in neurons (28). Conversely, Bak-deficient (Bak^{-/-}) mice have developmentally normal brains, suggesting that Bak is not important for developmental apoptosis in neurons (17), nor is it necessary for mitochondrial cytochrome *c* release in growth factor withdrawal in Bak^{-/-} neurons (30). Neurons may express a neuronal splice variant of Bak that acts as a proapoptotic BH3-only protein in a Bax-dependent manner (31). This explains why Bax-deficient neurons have defects in cytochrome *c* release and apoptosis in multiple models of apoptosis, including reovirus infection (6), while Bak-deficient neurons do not (30). The data reported here show that Bak plays a pathogenic role during reovirus encephalitis, although it still remains to be determined whether Bak functions in isolation from Bax or whether it acts to promote Bax activation.

We used pifithrin μ to show that inhibition of p53 translocation to mitochondria is important for reovirus pathogenesis in the CNS, as it blocks both reovirus-induced apoptosis and tissue injury. Pifithrin μ inhibits p53 binding to mitochondria by reducing its affinity for the antiapoptotic proteins Bcl-2 and Bcl-XL (22) while having no effect on the transactivational or cell cycle checkpoint control function of p53. Our results suggest that pifithrin μ also blocks p53 binding to Bak; however, whether this is a direct block or whether it occurs as a consequence of the inhibition of mitochondrial translocation of p53 remains to be determined.

Although our results show that pifithrin μ blocks p53 binding to Bak at the mitochondria, it is important to note that pifithrin μ may also affect interactions between the antiapoptotic protein Hsp70 (32) and its substrates, including apoptotic protease-activating factor 1 (Apaf-1) and apoptosis-inducing factor (AIF). In both these cases, pifithrin μ would be expected to increase apop-

FIG 6 Pifithrin μ reduces reovirus-induced caspase 3 activity and tissue injury in BSCs. Brain slices, prepared from 2- to 3-day-old SW mice, were infected with T3A reovirus (Reo) and treated with pifithrin μ ($P\mu$), as indicated. Infected slices treated with vehicle (veh) alone were used as controls. (A) Slices were harvested after treatment and assayed for apoptosis by using a fluorogenic caspase 3 activity assay. The graph shows the average fluorescence (arbitrary units) from 3 independent experiments and demonstrates that (i) infection of BSCs with reovirus results in an increase in caspase 3 activity and (ii) treatment with pifithrin μ significantly inhibits reovirus-induced activation of caspase 3. (B) Reovirus-induced tissue injury was determined by measuring LDH levels in the medium from the same slices. The graph shows the average absorbance (arbitrary units) and demonstrates that (i) infection of BSCs with reovirus results in an increase in tissue injury and (ii) treatment with pifithrin μ inhibits reovirus-induced tissue injury. (C) Bak-NT was used to immunoprecipitate proteins from reovirus-infected, pifithrin μ -treated BSCs. Western blotting demonstrates that pifithrin μ inhibits the formation of p53/Bak complexes in reovirus-infected BSCs. VDAC was used as an input protein loading control.

toxicity (33, 34). Hence, the effects of pifithrin μ on caspase activation and cell death in reovirus-infected *ex vivo* slice cultures are unlikely to be due to its inhibition of HSP70 interactions. Pifithrin μ can also inhibit the degradation of I κ B, leading to the inhibition of NF- κ B activation (35).

Our results suggest that there is a small discrepancy between caspase inhibition (1.5-fold) and LDH (2-fold). We suspect that the presence of p53 at mitochondria influences both caspase-dependent (via its interactions with Bcl-2 family proteins) and -independent mechanisms of cell death (36–38).

In summary, the work presented here demonstrates that the mitochondrial translocation of p53 during reovirus encephalitis, and its subsequent binding to Bak, is an important pathogenic mechanism. Our work substantiates our previously reported findings that mitochondrial apoptotic signaling contributes to reovirus-induced CNS disease and suggests that mechanisms designed to target mitochondrial signaling may have therapeutic potential for viral encephalitis.

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