The function of the cellular prion protein (PrP) is still poorly understood. We present here an unprecedented role for PrP against Bax-mediated neuronal apoptosis and show that PrP potently inhibits Bax-induced cell death in human primary neurons. Deletion of four octapeptide repeats of PrP (PrPΔOR) and familial DI78N and T183A PrP mutations completely or partially eliminate the neuroprotective effect of PrP. PrP remains anti-apoptotic despite truncation of the glycosylphosphatidylinositol (GPI) anchor signal peptide, indicating that the neuroprotective form of PrP does not require the abundant cell surface GPI-anchored PrP. Our results implicate PrP as a potent and novel anti-apoptotic protein against Bax-mediated cell death.

Prion protein (PrP) \(^1\) is a single-glycoprotein that is highly expressed in brain, heart, lungs, and lymphoid system and at lower levels in several other tissues such as muscle (1, 2). Mature PrP contains two N-linked glycans and a disulfide bond (reviewed in Ref. 3). PrP possesses a C-terminal GPI-anchoring signal and a transmembrane domain that can generate type I (**C**tmPrP) or type II (**N**tmPrP) transmembrane-spanning isoforms in isolated endoplasmic reticulum microsomes or phospholiposomes (4–7). In most cells, the majority of the PrP localizes to the cell surface as a GPI-anchored protein (8). The complete translocation of PrP is dependent on translocation accessory factors (TrAF). In the absence of TrAF, PrP is exclusively synthesized in a transmembrane topology (7).

Whereas the role of the infectious form of PrP in a number of human and animal neurodegenerative diseases has been extensively studied, the normal function of PrP is still poorly understood. PrP-null mice display no dramatic phenotype (9). Furthermore, four identical N-terminal PrP octapeptide repeats (OR) that are highly conserved in evolution share limited similarity with the Bcl-2 homology domain 2 (BH2) of Bcl-2 proteins (23, 24). Bcl-2 proteins are central to the regulation of cell death, and the BH2 domain is crucial to the anti-apoptotic function of Bcl-2 and its interaction with the pro-apoptotic Bax protein (24, 25). Based on these features of PrP, we hypothesized that similar to Bcl-2 family members, PrP may play a role in the regulation of neuronal apoptosis. In the present study, we have investigated the role of PrP in neuronal survival against the pro-apoptotic Bax protein.

**EXPERIMENTAL PROCEDURES**

**Clones and Antibodies—**Human Bcl-2 cDNA was a kind gift from Dr. Walter Nishioka (Vical Inc. San Diego, CA). PrP and PrP-D178N and -T183A DNAs were PCR-amplified as described (26, 27). Bax-\(\alpha\) cDNA was PCR-amplified from human neuron cDNA. cDNAs were cloned into pBluescript KSII (pBSK−; Stratagene) or pCep4β (Invitrogen). The APP\(_{695}\) construct has been described elsewhere (28). The PrP antisense construct (PrPAS) was made by cloning the entire PrP coding region in pBluescript KSII (pBSK−; Stratagene) or pCep4β (Invitrogen). Deletion of the OR region was performed by PCR amplification of the two regions flanking the ORs using the following primers. To generate the 5′-fragment: forward (PDG2), 5′-TACTGAGAATTCGCAGTCCATTATG-GGGAACCTTGGCTGTTG-3′ and reverse (PDG3), 5′-ACCCACCGCCC-GAGGGG′; to generate the 3′-fragment: forward (P4+), 5′-ACCCACAGTTCA-3′ and reverse (PDG1), 5′-GTACGTGGATCTCTCCTCA-TCCCCTACTACGGAGA′. The two fragments were then blunt-end ligated and cloned in the proper orientation in pBSK− and pCep4β. Deletion of the GPI anchor signal was generated by PCR using PDG2 (forward) and reverse 5′-TGGGGATCTCCATCTCCGATGG-3′ primers. R155 PrP antisemur against residues 36–56 was raised in our laboratory.

**Human Neurons and Microinjections—**Human primary neurons were cultured as described (29) and microinjected with 25 pl containing 0.75 pg of DNA and 2.5 pg of dextran Texas Red (DTR) in phosphate-buffered saline (30). Each injection was done on 200 neurons in at least three independent neuronal preparations. The cells were fixed in 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline. Cell death was assessed using the in situ cell detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The cells were fixed for 24 h and stained with DAPI and TUNEL. For the TUNEL assay, the percentage of cell death was determined as the number of TUNEL and DTR double-positive over the total number of DTR-microinjected neurons.

**Immunofluorescence with R155 PrP Antiserum—**Neurons were fixed in 4% paraformaldehyde, 4% sucrose 24 h after microinjection of the copper concentration, intracellular calcium concentration, activation of lymphocytes, astrocyte proliferation, and signal transduction and has antioxidant properties (11–16). Although controversial, PrP-null mice are also found to be impaired in long term potentiation (17–19). In addition, it has been shown that PrP-null neuronal cell lines are more susceptible to serum deprivation-induced cell death and that Bcl-2 overexpression can attenuate the sensitivity of PrP-null neuronal cell lines to serum deprivation (20). Kurschner and Morgan (21, 22) reported that yeast PrP fusion proteins interact with Bcl-2. Furthermore, four identical N-terminal PrP octapeptide repeats (OR) that are highly conserved in evolution share limited similarity with the Bcl-2 homology domain 2 (BH2) of Bcl-2 proteins (23, 24). Bcl-2 proteins are central to the regulation of cell death, and the BH2 domain is crucial to the anti-apoptotic function of Bcl-2 and its interaction with the pro-apoptotic Bax protein (24, 25). Based on these features of PrP, we hypothesized that similar to Bcl-2 family members, PrP may play a role in the regulation of neuronal apoptosis. In the present study, we have investigated the role of PrP in neuronal survival against the pro-apoptotic Bax protein.

**Prion Protein Protects Human Neurons against Bax-mediated Apoptosis**

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‡‡ The abbreviations used are: PrP, prion protein; GPI, glycosylphosphatidylinositol; OR, octapeptide repeats; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; TrAF, translocation accessory factors; FITC, fluorescein isothiocyanate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BFA, brefeldin A; ANOVA, analysis of variance; DTR, dextran Texas Red; PCR, polymerase chain reaction; APP, amyloid precursor protein; Bax, Bcl-2-associated protein x.
antisense PrP construct. The cells were permeabilized with 0.1% Triton X-100, blocked with 10% fetal goat serum, incubated with R155 (1:100), and detected with goat anti-rabbit IgAll-FITC.

Transfection of Erythroblleukemia K562 and M17 Neuroblastoma Cell Lines and Detection of Cep4β Construct Expression—Cells were grown to 70–80% confluence in 6-well plates and transfected with 3 μg of plasmid DNA using the LipofectAMINE 2000 reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Transient transfectants were assessed for protein expression at 48 h post-transfection. Stable cell lines for each construct were also studied. Cells were metabolically labeled for 4 h using 100 μCi/ml [35S]methionine. Cells were lysed in Nonidet P-40 lysis buffer, and PrP was immunoprecipitated in radioimmune precipitation buffer using anti-PrP R155 (31). The immune complexes were separated on Tri-Tricine gels and subjected to autoradiography.

Functional Assays in the Presence of Brefeldin A (BFA) and Monensin—Human primary neurons were preincubated for 1 h in the presence or absence of 5 μg/ml brefeldin A or 5 μM monensin (Sigma), microinjected as described above, and then incubated in the absence or presence of either 5 μg/ml brefeldin A or 5 μM monensin for 12 h. The cells were fixed at the indicated times, and cell death was assessed as described above. The effect of brefeldin A on prion maturation and secretion was studied in neuronal cultures incubated in serum- and methionine-free media in the presence of 5 μg/ml brefeldin A for 1 h and then incubated in 100 μCi/ml of [35S]methionine for 1, 3, and 12 h. Immunoprecipitations were done with a 1:100 dilution of polyclonal anti-PrP antisera as previously described (29).

Immunoprecipitations were done with a 1:100 dilution of polyclonal anti-PrP antisera R155 as previously described (29). Transient transfectants were assessed for protein expression at 48 h post-transfection. Stable cell lines for each construct were also studied. Cells were metabolically labeled for 4 h using 100 μCi/ml [35S]methionine. Cells were lysed in Nonidet P-40 lysis buffer, and PrP was immunoprecipitated in radioimmune precipitation buffer using anti-PrP R155 (31). The immune complexes were separated on Tri-Tricine gels and subjected to autoradiography.

RESULTS

PrP Protects Human Neurons against Bax-mediated Cell Death—PrP, Bax, and Bcl-2 are normally expressed in human neurons. However, Bax is not pro-apoptotic unless it is induced through insult or overexpression. We chose to induce Bax-mediated cell death by microinjecting of Bax cDNA because cell death can then be directly attributed to Bax overexpression. The role of PrP and Bcl-2 against Bax-mediated cell death was then assessed by co-microinjecting the cDNAs encoding Bcl-2 and PrP with the Bax cDNA in human primary neurons in culture. The cDNAs were expressed under the cytomegalovirus promoter of the episomal Cep4β construct (32). Bax is known to cause apoptosis in a number of neuronal cells (33, 34). Injection of Bax cDNA, but not vector pCep4β, Bcl-2, or PrP cDNAs, also induces a rapid cell death in 90% of these human primary neurons within 48 h (Fig. 1A). Co-injection of Bax cDNA with either PrP or Bcl-2 cDNA protects against Bax-mediated cell death. The triple injection of PrP, Bcl-2, and Bax does not further enhance protection. In contrast, microinjection of a cDNA encoding amyloid precursor protein (APP) does not prevent Bax-mediated cell death. These results show that PrP and Bcl-2 can both efficiently prevent Bax-mediated cell death in human neurons.

Endogenously Expressed PrP Protects against Bax-mediated Cell Death—to test if the neuroprotective function of PrP is merely a consequence of overexpression, endogenous PrP expression was inhibited by injecting neurons with a PrPAS cDNA Cep4β episomal construct. This construct has previously been used successfully to inhibit high levels of APP expression (35). The antisense construct did not induce cell death in the absence of Bax cDNA (Fig. 1B). However, the antisense cDNA enhanced Bax-mediated neuronal cell death at 12 and 24 h of injection. We confirmed that PrP expression decreases in the microinjected neurons (Fig. 2A, inset). Therefore, endogenous PrP can protect to some extent against Bax-mediated cell death. The antisense study also indicates that the loss of PrP expression is not a problem for these neurons unless Bax pro-apoptotic properties have been launched.

Deletion of the PrP Octapeptide Repeat Abolishes the Neuroprotective Function of PrP—The PrP octapeptide repeat region plays an important role against oxidative stress (15, 16). In addition, an increase in the number of OR in PrP leads to disease in human and mice (36, 37). To determine whether PrP anti-Bax function also requires the octapeptide repeat region, we tested the neuroprotective ability of PrP lacking the four OR with similarity to BH2 (PrPΔOR) against Bax. PrPΔOR abolishes the neuroprotective function of PrP (Fig. 2A). The lack of function of PrPΔOR cDNA construct could be caused by the absence of expression of this mutant PrP. Alternatively, the protein could be expressed but unstable. Because neurons are microinjected, we can only assess the expression of the exogenous PrP by immunocytochemistry. However, neurons express considerable levels of PrP, and it is therefore impossible to test the amount of proteins expressed from the Cep4β constructs in the microinjected neurons. Assuming that recombinant PrP is expressed similarly in neurons and other eukaryotic cells, we verified the expression level of PrP in eukaryotic cells by trans-

Fig. 1. PrP protects against Bax-mediated cell death in human neurons. A, survival of human neurons microinjected with dextran Texas Red and eukaryotic episomal pCep4β constructs expressing human Bax, Bcl-2, or PrP protein at 0, 24, and 48 h after injection. Bax × PrP, Bax × Bcl-2, and Bax × Bcl-2 × PrP are significantly different from Bax at 24 and 48 h (**, p < 0.01). B, survival of human neurons expressing human PrP, PrP antisense (PrPAS), or Bax at 0 (column 1), 12 (column 2), or 24 (column 3) hours after injection. Bax × PrPAS is significantly different from Bax (*, p < 0.05 and **, p < 0.01). The inset shows the inhibition of PrP expression by antisense PrP cDNA as verified in microinjected neurons (red) by immunocytochemistry to R155 (green). Immunofluorescence was not detected in the absence of primary antibody (not shown).
fecting erythroleukemia K562 and neuroblastoma M17, two cell lines that lack endogenous expression of PrP. The transfected cells were selected for hygromycin resistance and PrP expression verified by immunoprecipitation of PrP from protein extracts of \(^{[35S]}\)methionine metabolically labeled cells. We find that PrPΔOR is expressed at levels comparable with those of wild type PrP (Fig. 2B). Therefore, the loss of function of PrP is not likely a consequence of low PrPΔOR expression levels but rather the result of the disruption of the OR region.

**Brefeldin A and Monensin Treatment of Neurons Inhibit the PrP Neuroprotective Function**—To assess the location of the neuroprotective PrP, PrPΔ- and Bax-microinjected neurons were treated with the Golgi-disaggregating agent, BFA or monensin, an ionophore that prevents trafficking of secreted proteins past the cis-Golgi (38). BFA and monensin did not alter Bax-mediating cell death but completely inhibited the neuroprotective function of PrP against Bax (Fig. 2C). In normal neurons, a small amount of PrP is immunoprecipitated from secreted medium after 12 h indicating that BFA has effectively prevented the trafficking of PrP into the Golgi apparatus. Monensin has an effect on PrP that is similar to that of BFA. No mature protein is observed even after 12 h of labeling. However, monensin also reduces the level of PrP expression raising the additional possibility that with monensin treatment, low expression of PrP cannot compensate for the pro-apoptotic effect of Bax overexpression. In normal neurons, a small amount of PrP is immunoprecipitated from secreted medium after 12 h of labeling. However, this released PrP is not observed in BFA- or monensin-treated neurons. These results indicate that trafficking past the cis-Golgi is required for the neuroprotective function of PrP.

**The Cell Surface GPI-anchored Protein Is Not the Neuroprotective Form of PrP**—In most cells, the majority of PrP exists as a GPI-anchored cell surface protein while Bax is a cytosolic protein with a C-terminal hydrophobic membrane anchor (8, 39). To determine whether the GPI-anchored PrP mediates the neuroprotective function through signal transduction (14), we compared the neuroprotective ability of a PrP mutant lacking the GPI-anchor signal peptide sequence (PrPΔGPI) versus wild type PrP against Bax-induced neuronal apoptosis. PrPΔGPI is expressed at much lower levels than wild type PrP but is also secreted in the medium (Fig. 2B). However, PrPΔGPI is still neuroprotective (Fig. 2E) indicating that the cell surface GPI-anchored PrP is not necessary for PrP’s neuroprotective function.

**Loss of the Neuroprotective Function of PrP with the T183A and D178N Mutants**—We additionally tested if C-terminal mu-
In the present manuscript, we show an unprecedented function for prion protein against Bax-mediated neuronal cell death. Whereas the T183A Familial Atypical Spongiform Encephalopathy (FASE) mutation (26) partially inhibits PrP function, the D178N Fatal Familial Insomnia (FFI) mutation (27) completely abolishes the PrP neuroprotective function against Bax. These results indicate that the loss of function of PrP may be involved in the pathophysiology of these two diseases.

DISCUSSION

In the present manuscript, we show an unprecedented function for prion protein against Bax-mediated neuronal cell death. Whereas the infectious nature of PrP is extensively studied, little is known about its normal cellular function. Kuwahara et al. (20) have shown that neuronal cell lines derived from PrP-null mice were more susceptible to serum deprivation and could be rescued by Bcl-2 or PrP. However, the mechanism by which PrP protected these cells was not proposed. Here, we show that PrP can prevent Bax-mediated cell death to levels equal to the neuroprotective function of Bcl-2. The neuroprotective effect of PrP is strong and prevents almost all Bax-microinjected neurons from cell death. It has previously been demonstrated that PrP protects neurons against oxidative stress through the octapeptide repeats (16). Similarly, we find that deletion of the octapeptide repeats eliminates the neuroprotective function of PrP against Bax. However, PrP protects only 30% of cells against oxidative stress (15, 40) compared with almost 100% protection against Bax.

The neuroprotective function of normal PrP against Bax is surprising and unexpected. Generally, negative functions have been attributed to the PrP. However, a natural neuroprotective function is reasonable. First, PrP is highly expressed in some neurons and therefore would be expected to have a beneficial function. Second, the presence of endogenous PrP appears to be neuroprotective in vivo. For example, the presence of endogenous mouse PrP allows the production of hamster scrapie PrP in GFAP-hamster PrP transgenic mice infected with the hamster prion strain, 263K. In addition, transmission of disease is obtained from these mice. However, the prnp+/− mice do not develop the neurodegenerative disorder observed in mouse prnp-null/GFAP-hamster PrP transgenic mice (41). Although reduced titers (10–100-fold) of the PrP proteinase K-resistant form and infectivity in the mouse prnp+/−/transgenic hamster PrP could explain the lack of neurotoxicity, an alternative possibility is that the endogenous PrP is neuroprotective against the presence of the scrapie isoform of the PrP. PrP also protects against the cytotoxic effects of Doppel in Purkinje neurons (42). Bax is a powerful executioner of neurons (33, 34). We find that this is also true in these human primary neurons. Although microinjection of recombinant active caspases leads to a slow death of the human primary neurons (30), Bax induces TUNEL-positive cell death in almost 70% of cells within 12 h (Fig. 1B). Therefore, PrP may be a natural strong protector against a major neuronal pro-apoptotic protein.

Our finding that the GPI anchor is not required for PrP’s anti-Bax function is also quite surprising. It is well established that the cell surface GPI-anchored PrP is the most abundant isoform of PrP in most cells. The results of the BFA experiment show that the neuroprotective form of PrP requires post-cis-Golgi modification or trafficking for function. However, with deletion of the GPI sequence, which abolishes the possibility of the GPI anchoring of PrP, the function is retained. Therefore, we conclude that the cell surface GPI-anchored PrP is not necessarily required for the neuroprotective function of PrP against Bax and that either a secreted or transmembrane form of PrP can embody the neuroprotective function.

Interestingly, we find that the FFI and FASE PrP mutations undergo a loss of function against Bax-mediated cell death. Whether this is because of altered protein conformation or improper trafficking will need to be determined. However, these results suggest a possible mechanism to account for the extensive neuronal loss in these two neurodegenerative diseases.

The unusual transmissible features of prion diseases have led to research where the major emphasis is on the “disease-causing” forms of PrP. Therefore, our finding that PrP is actually a strong neuroprotective agent against the major pro-apoptotic Bax protein is counterintuitive. We require further work in order to decipher the underlying molecular mechanism of PrP function against Bax. However, this study shows an interesting twist regarding the function of the already quite fascinating prion protein.

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