Several lines of evidence suggest that PrPC, the non-infectious form of the prion protein, may function to protect neurons and other cells from stress or toxicity. In this paper, we report on the use of the yeast Saccharomyces cerevisiae as a model system to assay the cytotoxic activity of PrPC. The mammalian pro-apoptotic protein, Bax, confers a lethal phenotype when expressed in yeast. Since overexpression of PrPC has been found to prevent Bax-mediated cell death in cultured human neurons, we explored whether PrPC could also suppress Bax-induced cell death in yeast. We utilized a form of mouse PrP containing a modified signal peptide that we had previously shown is efficiently targeted to the secretory pathway in yeast. We found that this PrP potently suppressed the death of yeast cells expressing mammalian Bax under control of a galactose-inducible promoter. In contrast, cytosolic PrP-(23–231) failed to rescue growth of Bax-expressing yeast, indicating that protective activity requires targeting of PrP to the secretory pathway. Deletion of the octapeptide repeat region did not affect the rescuing activity of PrP, but deletion of a charged region encompassing residues 23–31 partially eliminated activity. We also tested several PrP mutants associated with human familial prion diseases and found that only a mutant containing nine extra octapeptide repeats failed to suppress Bax-induced cell death. These findings establish a simple and genetically tractable system for assaying a putative biological activity of PrPC.

Prion diseases are a group of transmissible neurodegenerative disorders, including Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, kuru, and fatal familial insomnia in human beings, as well as scrapie and bovine spongiform encephalopathy in animals (1). These diseases are caused by the conversion of PrPSc, a normal cell surface glycoprotein, into PrPSc, a β-sheet-rich conformer that is infectious in the absence of nucleic acid (2, 3). Although a great deal is known about the role of PrPSc in the disease process, the normal function of PrPC has remained elusive. A variety of functions have been proposed for PrPC, including roles in metal ion trafficking (4), cell adhesion (5), and transmembrane signaling (6). Identifying the function of PrP may provide important clues to the pathogenesis of prion diseases, since there is evidence that PrPC plays an essential role in mediating the neurotoxic effects of PrPSc (7).

Several intriguing lines of evidence have emerged recently indicating that PrPC may function to protect cells from various kinds of internal or environmental stress (8). For example, PrP overexpression rescues cultured neurons and some mammalian cell lines from pro-apoptotic stimuli, including Bax expression, serum withdrawal, and cytokine treatment (9–12). In addition, PrP-null mice are more susceptible to neuronal loss after experimental brain injury (13), and neurons cultured from these animals display abnormalities related to increased susceptibility to oxidative stress (14). Finally, expression of wild-type PrP completely abrogates the neurodegenerative phenotype of mice expressing the PrP parologue, Doppel, or N-terminally truncated forms of PrP (Δ32–121 and Δ32–134) (15, 16).

In this paper, we have utilized the yeast Saccharomyces cerevisiae as a experimentally accessible model system in which to analyze the cytoprotective function of PrPC. In particular, we tested whether PrP could rescue yeast from cell death induced by the pro-apoptotic protein, Bax, similar to the way PrP has been shown to protect cultured human neurons from Bax-induced death (9, 12). We took advantage of the fact that heterologous expression of mammalian Bax in yeast is lethal (17), a phenomenon that has been used as an assay to isolate Bax inhibitor proteins from mammalian sources (18–20). We also capitalized on our recent demonstration that PrP with a modified signal peptide is efficiently targeted to the yeast secretory pathway, where it becomes glycosylated, glycolipid-anchored, and localized to the plasma membrane as it does in mammalian cells (21). We show here that PrP targeted to the secretory pathway efficiently suppresses Bax-induced cell death in yeast, and we perform a structure-function analysis to determine which features of the PrP molecule are essential for this effect. Our results establish a simple and genetically tractable system for assaying a putative biological activity of PrPC.

**EXPERIMENTAL PROCEDURES**

**Yeast—Strain YPH499 (MATa ura3–52 lys2–801 ade2–101 trp1–143 his3–Δ200 leu2–3,112) was obtained from Stratagene (La Jolla, CA). Yeast were grown at 30°C on SD (synthetic medium containing glucose as the sole carbon source) or SG (synthetic medium containing galactose as the sole carbon source), with drop-out of either Ura, Leu, or both.**

**Plasmids—Murine Bax was expressed using the vector pESC-LEU (Stratagene, La Jolla, CA), which contains a 2-micron origin of replication, two galactose-inducible promoters (GAL10 and GAL1), and a leucine (Leu) selection marker.**

The murine Bax coding sequence was amplified by PCR from a full-length cDNA clone (IMAGE 3968903; Open Biosystems, Huntsville, AL) using the following primers: 5'-TA-
TAATTAAGCTGGCCGATGAGGTCCGGGAG-3' (forward) and 5'-CCGCCTGTTAGCTTCAAGCCCATTTTCCTCCAGATGGT-3' (reverse). The forward primer includes a NotI restriction site, and the reverse primer includes a BglII restriction site. The NotI/BglII-digested PCR product was cloned into pESC-LEU that had been cut with the same two enzymes, which places the Bax coding region under control of the GAL10 promoter.

All PrP constructs were derived from the murine PrP sequence and contained the 3F4 epitope tag (22). All constructs were expressed using the vector p426GPD, which contains a 2-micron origin of replication, a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, and a uracil (Ura) selection marker (23). The coding region of DPAPB-PrP254 was subcloned from pVT102U into p426GPD using BamHI and HindIII sites. The construction DPAPB-PrP254/pVT102U has been described previously (21).

Deletion and truncation mutants of PrP (Δ51–90, Δ32–80, Δ32–93, Δ23–31, 23–231, Δ231–254, GPI-) were generated by bridge PCR using DPAPB-PrP254 as a template. PCR products were then cloned into p426GPD via BamHI and HindIII sites. Point and insertional mutants of PrP (PG14, E199K, P101L, D177N, F197S) were created by removing a KpnI/XbaI fragment from DPAPB-PrP254/pVT102U and substituting the equivalent fragment excised from previously constructed pcDNA3 plasmids carrying these mutations (24). The DPAPB-PrP254 coding regions were then subcloned from pVT102U into p426GPD via BamHI and HindIII sites.

The coding region of murine BI-1 (18) with a single C-terminal HA tag (YPYDVPDYA) was amplified by PCR from a full-length cDNA clone (IMAGE 3708463; Open Biosystems) and was cloned into p426GPD via BamHI and HindIII sites.

Yeast cells were transformed by electroporation (25).

**Protein Analysis**—Proteins were extracted from yeast by lysis in NaOH/β-mercaptoethanol as described previously (26) and were resolved using DPAPB-PrP254 as a template. PCR products were then cloned into pESC-LEU via BamHI and HindIII sites.

**RESULTS**

**PrP Rescues Yeast from Bax-induced Cell Death**—We expressed murine Bax in *S. cerevisiae* using a galactose-inducible promoter (GAL10), so that growth in the presence or absence of Bax could be assessed by plating on galactose medium (SG) or glucose medium (SD), respectively. Overnight cultures were serially diluted and spotted on both kinds of plates. Yeast carrying the inducible Bax plasmid grew well on SD-Leu but failed to grow on SG-Leu (supplemental Fig. S1A). In contrast, yeast transformed with empty vector grew similarly on both plates. Western blotting confirmed that growth in galactose medium induced expression of Bax (supplemental Fig. S1B). Thus, inducible expression of Bax is lethal in yeast.

To test whether mammalian PrP could rescue yeast from Bax-induced cell death, we utilized DPAPB-PrP254, a form of PrP containing a chimeric signal peptide consisting of the signal-anchor sequence of the yeast protein DPAPB fused to the C-terminal 7 amino acids of the PrP signal peptide. We have shown previously that DPAPB-PrP254 is efficiently targeted to
plates as described in the legend to Fig. 1. Serial dilutions of cultures of three independent transformants (a–c) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.

The secretory pathway in yeast, where it is glycosylated, GPI-anchored, and trafficked to the plasma membrane (21). Bax-expressing yeast were transformed with a plasmid that expresses DPAPB-PrP254 under control of the strong, constitutive GPD promoter. As controls, yeast were transformed with empty vector or with the same vector encoding murine Bax inhibitor 1 (BI-1), which has previously been shown to rescue yeast from Bax-induced cell death (18). Over-night cultures of three independent transformants of each strain were serially diluted and spotted on glucose (SD-Leu-Ura) and galactose (SG-Leu-Ura) plates.

Each of the strains grew on glucose plates, although yeast expressing DPAPB-PrP254 were slightly inhibited in their growth compared with the other two strains (Fig. 1A). On galactose plates, as expected, vector-transformed yeast failed to grow, while yeast expressing BI-1 were partially rescued. Remarkably, yeast expressing DPAPB-PrP254 grew even better on galactose than yeast expressing BI-1. Quantitation of the number of colonies on galactose plates compared with glucose plates revealed that DPAPB-PrP254 rescued 80% of the growth in the presence of Bax, compared with 50% for BI-1 and 2% for empty vector (Fig. 1B). We also noticed that PrP-expressing yeast produced larger colonies than BI-1-expressing yeast on galactose plates (Fig. 1A), implying that PrP is more potent than BI-1 in enhancing growth rate as well as increasing the number of surviving cells in the presence of Bax. Western blotting confirmed expression of Bax, DPAPB-PrP254, and BI-1 in yeast strains grown in galactose medium, demonstrating that DPAPB-PrP254 and BI-1 do not interfere with expression of Bax (Fig. 1C). The data shown in Fig. 1 were obtained using the yeast strain YPH499, but similar results were obtained with strain BY4741 (data not shown).

We performed two additional control experiments. To exclude the possibility that the expression of DPAPB-PrP254 enhanced growth on galactose medium independent of Bax, we analyzed yeast expressing only DPAPB-PrP254 without Bax. We found that expression of DPAPB-PrP254 did not enhance, and actually slightly inhibited, growth on SG-Ura, when comparison was made to yeast transformed with empty vector (supplemental Fig. S2A). To confirm that the growth rescuing effect was due to the presence of the DPAPB-PrP254 plasmid and not to another genetic alteration, we selected for yeast that had lost this Ura-marked plasmid by growth on 5-fluoroorotic acid. We found that yeast that had lost the plasmid did not retain the ability to grow on galactose medium (supplemental Fig. S2B).

Yeast expressing and actually slightly inhibited growth on SG-Ura, when compared with the other two strains (Fig. 1A). On galactose plates, as expected, vector-transformed yeast failed to grow, while yeast expressing BI-1 were partially rescued. Remarkably, yeast expressing DPAPB-PrP254 grew even better on galactose than yeast expressing BI-1. Quantitation of the number of colonies on galactose plates compared with glucose plates revealed that DPAPB-PrP254 rescued 80% of the growth in the presence of Bax, compared with 50% for BI-1 and 2% for empty vector (Fig. 1B). We also noticed that PrP-expressing yeast produced larger colonies than BI-1-expressing yeast on galactose plates (Fig. 1A), implying that PrP is more potent than BI-1 in enhancing growth rate as well as increasing the number of surviving cells in the presence of Bax. Western blotting confirmed expression of Bax, DPAPB-PrP254, and BI-1 in yeast strains grown in galactose medium, demonstrating that DPAPB-PrP254 and BI-1 do not interfere with expression of Bax (Fig. 1C). The data shown in Fig. 1 were obtained using the yeast strain YPH499, but similar results were obtained with strain BY4741 (data not shown).

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Cytosolic PrP Does Not Suppress Bax-induced Cell Death, but Non-membrane-anchored PrP Does—To investigate the rescuing activity of cytosolic PrP, Bax-expressing yeast were transformed with a plasmid encoding PrP23–231. This protein lacks both the N-terminal signal peptide and the C-terminal GPI addition signal and would therefore be expected to be localized to the yeast cytosol. We found that yeast expressing PrP23–231 failed to grow on SG-Leu-Ura medium, similar to vector-transformed yeast, indicating that cytosolic PrP is incapable of rescuing yeast from Bax-induced cell death (Fig. 2). We did not note any difference in growth of the two strains on SD-Leu-Ura, demonstrating that cytosolic PrP is not toxic in the absence of Bax. Western blots confirmed that PrP23–231 was being expressed and that, in contrast to DPAPB-PrP254, it was not glycosylated, consistent with failure to enter the secretory pathway (supplemental Fig. S3A).

To test whether GPI anchoring to the cell membrane was essential for the rescuing activity of PrP, we tested yeast transformed with a plasmid encoding DPAPB-PrP230, which contains the chimeric signal sequence but is missing the C-terminal GPI addition signal. This form of PrP displayed full rescue activity of cytosolic PrP, Bax-expressing yeast were transformed with empty p426GPD vector (Vector) or with p426GPD vector encoding DPAPB-PrP254 (WT-PrP) or DPAPB-PrP230 (PrP GPI–). Serial dilutions of cultures of three independent transformants (a–c) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.

Deletion of residues 23–31, but not the octapeptide repeats, reduces rescue activity. Yeast expressing Bax under control of a galactose-inducible promoter were transformed with empty p426GPD vector (Vector) or with p426GPD vector encoding DPAPB-PrP254 (WT-PrP) or DPAPB-PrP230 carrying the indicated deletions (Δ51–90, Δ32–80, Δ32–93, and Δ23–31). Serial dilutions of cultures of three independent transformants (a–c) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.

Cytosolic PrP Suppresses Bax-induced Cell Death in Yeast

PrP Suppresses Bax-induced Cell Death in Yeast

The data shown in Fig. 1 were obtained using the yeast strain YPH499, but similar results were obtained with strain BY4741 (data not shown).

FIG. 2. Cytosolic PrP does not suppress Bax-induced cell death, but non-membrane-anchored PrP does. Yeast expressing Bax under control of a galactose-inducible promoter were transformed with empty p426GPD vector (Vector) or with p426GPD vector encoding DPAPB-PrP254 (WT-PrP), PrP23–231, or DPAPB-PrP230 (PrP GPI–). Serial dilutions of cultures of three independent transformants (a–c) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.
cell death by mediating release of cytochrome c in response to apoptotic signals, where it promotes Bax, a cytoplasmic protein that is translocated to mitochondria. Of note, several mammalian proteins that were isolated as prion disease-related proteins play a role in the PrP rescue pathway we have demonstrated here. One hypothesis is that these molecules, which may include reactive oxygen species generated in mitochondria, may involve PrP directly associates with and inactivates Bax. Although PrP was reported to interact with Bcl-2 in mitochondrial and was then transferred to SG-Leu-Ura medium for 18 h to induce Bax expression. Cell lysates were digested with the indicated amounts of PK, after which PrP was visualized by Western blotting using 3F4 antibody. Lanes 1, 5, 9, 13, 17, and 21 represent 200 μg of protein and the other lanes 800 μg of protein. The diglycosylated, monoglycosylated, and nonglycosylated forms of PG14 PrP, which are not well separated in lane 5, migrate at 37, 35, and 33 kDa, respectively. C, yeast were grown as described for B. Cell lysates were centrifuged at 186,000 × g for 45 min, after which PrP in supernatant (S) and pellet (P) fractions was analyzed by Western blotting using 3F4 antibody.

**DISCUSSION**

In this study, we have demonstrated that PrP targeted to the secretory pathway of *S. cerevisiae* is capable of rescuing the lethal phenotype caused by heterologous expression of Bax, a pro-apoptotic member of the *Bcl-2* gene family. In mammalian cells, Bax is a cytoplasmic protein that is translocated to mitochondria in response to apoptotic signals, where it promotes cell death by mediating release of cytochrome c, which in turn activates caspase-dependent pathways. Although *S. cerevisiae* does not contain endogenous *Bcl-2* family members or caspases, the initial events underlying Bax activity in yeast and mammalian cells are similar, including translocation of the protein to mitochondria, release of cytochrome c, and alterations in mitochondrial function. The downstream effectors of Bax-induced cell death in yeast are less certain, but may include reactive oxygen species generated in mitochondria. Of note, several mammalian proteins that were isolated by virtue of their ability to suppress Bax-induced cell death in yeast also have Bax inhibitory activity in mammalian cells (18–20). Thus, it is likely that the ability of PrP to rescue the lethal phenotype of Bax-expressing yeast reflects interaction with Bax-related pathways that are operative in higher organisms.

How does PrP protect yeast from Bax-induced cell death? One possibility is that PrP directly associates with and inactivates Bax. Although PrP was reported to interact with Bcl-2 in a yeast two-hybrid assay (31, 32), binding of DPAPB-PrP to Bax in our expression system seems unlikely, since DPAPB-PrP is present in the secretory pathway while Bax is in the cytoplasm. Indeed, when we deliberately targeted PrP to the cytoplasm by deletion of the N- and C-terminal signal sequences (PrP23–231), the protein lost its Bax rescuing activity. Whether the inactivity of cytosolic PrP is due to protein aggregation or to other factors remains to be determined. A second, more likely possibility to explain the protective effect of PrP is that PrP interacts with endogenous yeast proteins that lie downstream of Bax in a cellular stress or toxicity pathway. In addition to members of the *Bcl-2* family, several other mammalian proteins are known to inhibit the action of Bax (18–20), and it is possible that yeast homologues of these or related molecules play a role in the PrP rescue pathway we have demonstrated here.

The results reported here are relevant to theories about the pathogenicity of mutant PrP molecules associated with inherited prion diseases. One hypothesis is that these molecules...
acquire toxic properties by virtue of misfolding or being converted to a PrPSc-like state. Alternatively, the presence of the mutation may abrogate or alter a normal physiological function of PrPSc. We found that PrP molecules carrying a nine-octapeptide insertion (PG14), but not one of several point mutations, failed to suppress Bax-mediated cell death. In addition, PG14 PrP was the only one of the mutants that showed significant protease resistance and detergent insolubility when expressed in yeast. These results suggest that loss of Bax-protective function, perhaps as a result of protein aggregation or misfolding, could play some role in PG14-associated prion disease. This conclusion is consistent with our observation that neuronal death in Tg(PG14) mice is Bax-dependent (33). It is possible that this mechanism is also applicable to other pathogenic mutants, which display PrPSc-like biochemical properties in cultured mammalian cells (34) and brain (27), even though they do not in yeast.

PrP has also been reported to protect against cell death in several mammalian systems. The studies that are most directly comparable to ours are those in which human PrP was shown to prevent Bax-induced apoptosis of cultured human neurons, following microinjection of plasmids encoding these proteins (9, 12). In those studies, in contrast to ours, PrP containing the first octapeptide repeat, was inactive in neurons, but we did not test this construct in yeast. It is possible that the discrepancies between yeast and human neurons reflect fundamental mechanistic differences in how PrP suppresses Bax-induced cell death in the two systems. Alternatively, the pathways involved in PrP cytoprotection may be partially, but not completely, conserved between yeast and mammals. Interestingly, PrPΔ32–93, which rescues Bax-induced cell death in yeast, is also capable of rescuing neurodegeneration in transgenic mice expressing PrPΔ32–134 (15). Moreover, deletion of a charged region encompassing residues 23–31 partially abolishes PrP rescue activity in yeast, and a slightly smaller deletion within the same region (23–28) has been found to abrogate protection against Doppell-induced apoptosis in mouse neurons (35). Taken together, these comparisons suggest that the ability of PrP to suppress Bax lethality in yeast reflects a physiological activity of PrP that is operative in mammals and that may protect cells against several kinds of toxic insults.

The observations reported in this paper establish yeast as a valuable tool to assay a potentially important biological activity of PrP. This system offers many advantages, most importantly, the capability of genetic analysis of PrP function. With the use of appropriately designed genetic screens and selections, it should now be possible to identify proteins from yeast and mammals that alter the cytoprotective activity of PrP and that may also interact physically with PrP.

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