STRUCTURE-FUNCTION ANALYSIS OF THE ANTI-BAX
FUNCTION OF THE PRION PROTEIN

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ABSTRACT

The Prion Protein (PrP) prevents Bax activation in primary human neurons and in MCF-7 breast carcinoma cells. To identify the PrP elements necessary for this anti-Bax function, we performed structure-function analyses on cytosolic PrP (CyPrP). Partial but not complete deletion of the N-terminal BH2-like octapeptide repeats (BORs) abolish CyPrP’s anti-Bax function. Deletion of the third and last α-helix (PrP23-199) eliminates CyPrP’s protection against Bax-mediated cell death, as do further C-terminal deletions. Substitution of helix 3 residues K204, V210, and E219 by prolines inhibits the anti-Bax function of CyPrP while replacement of these three residues by alanines causes a loss of function only for the K204A mutant. Expression of PrP’s helix 3 displays anti-Bax activity in both MCF-7 cells and human neurons. Together, these results indicate that although the BOR domain is implicated, helix 3 is necessary and sufficient for the anti-Bax function of CyPrP. Identification of helix 3 as the structural element for the anti-Bax function thus provides a molecular target to modulate PrP’s anti-Bax function in cancer and neurodegeneration.
**RÉSUMÉ**

La protéine du prion (PrP) empêche l’activation de Bax dans les neurones humains primaires et dans les cellules de carcinome du sein MCF-7. Afin d’identifier les éléments de PrP requis pour cette fonction anti-Bax, nous avons étudié la relation structure-fonction de la forme cytosolique de PrP (CyPrP). La délétion partielle mais non complète d’une série d’octapeptides du fragment N-terminal semblables au domaine BH2 abolit la fonction anti-Bax de CyPrP. La délétion de la troisième et dernière hélice ainsi que des délétions plus importantes à partir du bout C-terminal éliminent également l’action protectrice de CyPrP contre la mort cellulaire médiée par Bax. Le remplacement des acides aminés de l’hélice 3 K204, V210 et E219 par des prolines inhibe la fonction anti-Bax de CyPrP. Lorsque ces trois acides sont remplacés par des alanines, seul le mutant K204A perd la fonction anti-Bax. L’expression de l’hélice 3 de PrP montre une activité anti-Bax chez les cellules MCF-7 et les neurones humains. Ensemble, ces résultats indiquent que malgré l’implication des octapeptides dans la fonction anti-Bax de PrP, l’hélice 3 est nécessaire et suffisante pour ce rôle protecteur de CyPrP. L’identification de l’hélice 3 comme élément-clé de la fonction anti-Bax de PrP donne une cible moléculaire pour moduler la mort cellulaire. Ainsi, cette hélice peut mener vers des avancées dans la lutte contre le cancer ou contre les maladies neurodégénératives.
PREFACE

This thesis examines the relationship between the structural elements of PrP and its protective function against Bax-mediated cell death in breast carcinoma MCF-7 cells and in human primary neurons. It has been divided in three chapters: the literature review, the original research data as submitted for publication, and the general discussion and future directions of the work.

The second chapter of the present thesis contains the following manuscript:

Stéphanie Laroche-Pierre, Julie Jodoin, and Andréa C. LeBlanc.

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ABBREVIATIONS

Apaf-1: Apoptotic protease activating factor-1
APC: Antigen-presenting cells
Bak: Bcl-2 homologous antagonist/killer
Bax: Bcl-2-associated X protein
BAR: Bifunctional apoptosis regulator
BCA: Bicinchoninic acid
Bcl-2: B cell lymphoma/leukemia 2
Bcl-xL: B cell lymphoma/leukemia extra-long protein
BH: Bcl-2 homology domain
BI-1: Bax inhibitor 1
Bid: BH3 interacting domain death agonist
Bip: Immunoglobulin heavy chain-binding protein
BOR: BH2-like octapeptide repeats
C. elegans: Caenorhabditis elegans
CD: Circular dichroism
cDNA: Complementary deoxyribonucleic acid
CJD: Creutzfeldt-Jakob disease
CMV: Cytomegalovirus
CNX: Calnexin
CRT: Calreticulin
CyPrP: Cytosolic Prion Protein
Doppel: Downstream prion protein-like
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EDEM</td>
<td>ER degradation enhancing alpha-mannosidase-like protein</td>
</tr>
<tr>
<td>EF-1a</td>
<td>Elongation factor 1-alpha</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Proteins</td>
</tr>
<tr>
<td>EGFP-Bax</td>
<td>Bax N-terminally tagged with EGFP</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-amino butyric acid</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat-shock protein</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
</tr>
<tr>
<td>NRAGE</td>
<td>Neurotrophin receptor interacting melanoma-associated antigen homolog</td>
</tr>
<tr>
<td>OR</td>
<td>Octapeptide repeat</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
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PI3 kinases: Phosphatidylinositol-3-kinases
PK: Proteinase K
PNGase: Peptide: N-glycosidase
PPI: Peptidylprolyl cis-trans isomerase
PrP: Prion protein
PrPSc: Scrapie form of PrP
PVDF: Polyvinylidene fluoride
RNAi: Ribonucleic acid interference
RPMI: Roswell Park Memorial Institute
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD: Superoxide dismutase
SP: N-terminal ER-targeting signal peptide
SRP: Signal recognition particle
STE: Stop-transfer effector (sequence)
STI-1: Stress-inducible protein-1
TFE: Trifluoroethanol
TM: Transmembrane domain
TNF: Tumor necrosis factor
TRAIL: TNF-related apoptosis-inducing ligand
TRAP: Translocon-associated protein
TSEs: Transmissible spongiform encephalopathies
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INTRODUCTION

PrP is best known for its involvement in Transmissible spongiform encephalopathies (TSEs), yet little attention is given to its protective role. This is unfortunate since better understanding of this function can have therapeutic applications against a variety of diseases including TSEs.

It has been found in our laboratory that PrP specifically inhibits Bax-mediated cell death, notably in human neurons and MCF-7 cells. The cytosolic form of PrP, and not the transmembrane forms or the more abundant GPI-anchored PrP, is responsible for stopping the conformational change that triggers Bax activation. The research presented in this thesis used structure-function analysis to find active and regulatory sites mediating PrP’s anti-Bax function in hope to decipher the mechanism by which PrP inhibits Bax activation.

Folded PrP has a flexible N-terminal tail that contains a series of octapeptide repeats (OR) and the transmembrane domain. The C-terminal portion is composed of three helices and a short beta-sheet arranged into a globular domain. Previous mutagenesis studies suggest the OR and globular domain play an important role in the anti-Bax function of PrP.

The work presented here used cell death assays on MCF-7 cells and human neurons overexpressing PrP deletion and substitution mutants alone or co-expressed with Bax. This allowed the identification of key domains for the anti-Bax function of PrP.
CHAPTER 1: LITERATURE REVIEW
The Prion Protein (PrP) is a cellular glycoprotein most highly expressed in the brain but also found in lymphoid tissue, heart and lungs (Oesch et al., 1985). Most studies on PrP focus on its involvement in transmissible spongiform encephalopathies (TSEs) rather than on its physiological function.

1. **PrP synthesis**

1.1 *Synthesis overview*

Human PrP is encoded by the *PRNP* gene on the short arm of chromosome 20 (Figure 1(1)) (Makrinou et al., 2002). As it is translated, PrP is targeted to the endoplasmic reticulum (ER) by an N-terminal signal peptide (SP) (Basler et al., 1986; Caughey et al., 1989; Robakis et al., 1986). This sequence mediates Translocon-associated protein (TRAP)-assisted entry through the Sec61 channel complex (Figure 1(2)) (Fons et al., 2003). The SP is cleaved off by a signal peptidase before complete PrP translocation into the ER (Hegde and Bernstein, 2006). Sequences in the C-terminus of PrP are also required for ER insertion (Heske et al., 2004). In *vitro*, even the glycosylphosphatidylinositol (GPI) anchor signal peptide can mediate translocation into the ER on its own (Hölscher et al., 2001).

As it enters the ER, PrP is folded by the chaperones immunoglobulin heavy chain-binding protein (Bip), a Hsp 70 family member (Jin et al., 2000), the peptidylprolyl cis-trans isomerase (PPI) cyclophilin (Cohen and Taraboulos, 2003), and the lectin calnexin (CNX) (Rudd et al., 2001). Once inside the ER, PrP is glycosylated at the two N-X-S/T consensus sites on asparagines 181 and 197 (Gavel and von Heijne, 1990; Haraguchi et al., 1989). PrP is then stabilized by
Figure 1. PrP synthesis overview.

Schematic diagram outlining the main steps of PrP synthesis and quality control.

1. PRNP gene transcription. 2. RNA export and co-translational translocation into the ER. 3. Glycosylation and disulfide bond formation. 4. Vesicular transport of the properly folded PrP to the Golgi apparatus. 5. Secreted PrP with and without the GPI anchor. 6. Retrotranslocation of PrP into the cytosol. 7. Protein degradation by the proteasome. 8. Direct PrP translation in the cytosol resulting in SP-CyPrP. Abbreviations: ER: endoplasmic reticulum. GPI: glycosylphosphatidylinositol anchor. SP: ER-targeting signal peptide.
the protein disulfide isomerase (PDI)-mediated formation of a disulfide bond between cysteines 179 and 214 (Figure 1(3)) (Molinari and Helenius, 1999; Turk et al., 1988). PrP is also cleaved at its C-terminus to allow the addition of the GPI anchor (Stahl et al., 1987). This addition also requires the region of the first helix of PrP (Winklhofer et al., 2003).

PrP continues through the classic secretory pathway to reach the Golgi apparatus by vesicular transport on the way to the plasma membrane (Figure 1(4)) (Taraboulos et al., 1992). In the Golgi, the oligosaccharides are further modified by addition of sialic acid (Harris, 2003; Tatzelt and Winklhofer, 2004). The end result of this pathway is a GPI-anchored plasma membrane PrP glycoprotein (Figure 1(5)).

PrP can adopt other topologies as transmembrane forms with either the N-terminus or the C-terminus facing the ER lumen have been observed. An anchor-less form of PrP can also be secreted into the extracellular medium (Figure 1 (5)) (Lin et al., 2008). Production of transmembrane or secreted forms of PrP depends on the SP and transmembrane domain as well as on the moment when the nascent chain is bound by chaperones and translocation proteins (Hegde et al., 1998a; Hegde et al., 1998b; Kim and Hegde, 2002; Kim et al., 2001; Yost et al., 1990). Topology can also depend on the lipid composition of the membranes (Schneiter and Toulmay, 2007). Aside from membrane-associated or secreted forms, PrP has also been found in the cytosol (Ma and Lindquist, 2001; Roucou et al., 2003; Yedidia et al., 2001; Zanusso et al., 1999).
1.2 Quality control and ERAD pathway

In the ER, PrP undergoes various quality control mechanisms to ensure its proper folding before proceeding to the cell surface (Ellgaard and Helenius, 2003). Quality control of secretory pathway proteins is done by cycles of addition and removal of a single glucose on the core asparagine-linked oligosaccharides. These cycles continue for as long as the native conformation is not achieved. The misfolded and monoglycosylated protein is bound by CNX or calreticulin (CRT). These chaperones attempt to fold the ER protein into the proper conformation after which the protein dissociates from the chaperones and is deglucosylated. Proteins remaining in a non-native conformation are recognized, re-monoglycosylated and rebound by the same chaperones (Ellgaard and Helenius, 2001; Parodi, 2000). Once the proteins are properly folded, they are deglucosylated and leave the cycle to proceed to the Golgi apparatus (Ellgaard and Helenius, 2003; Hammond et al., 1994). A protein that has gone through this cycle several times without achieving proper conformation is targeted for degradation by the removal of a mannose unit from its core oligosaccharides (Ellgaard and Helenius, 2003; Hosokawa et al., 2001; Parodi, 2000). These mannose-trimmed proteins are bound by the lectin ER degradation enhancing alpha-mannosidase-like protein (EDEM), which targets them to the ER-associated degradation (ERAD) pathway (Figure 1(6)) (Hosokawa et al., 2001).

Some quality control can also occur in the Golgi either through the return of proteins to the ER or through resident chaperones (Ellgaard and Helenius, 2003).
The ERAD pathway has four steps: recognition of the misfolded proteins and targeting for retrotranslocation, transport to the cytosol, release from the reverse translocation machinery, and degradation (Tsai et al., 2002).

Proteins to be degraded in the cytosol are recognized by ER chaperones through continuously exposed hydrophobic segments (Ellgaard and Helenius, 2003; Tsai et al., 2002). As mentioned previously, lectins such as EDEM direct the targeted proteins to their final destination (Hosokawa et al., 2001).

The ERAD-targeted proteins are thought to exit the ER via the Sec61 complex (Figure 1(6)) (Tsai et al., 2002; Wiertz et al., 1996). As they exit, proteins to be degraded are recognized by an E3 ligase, which adds ubiquitin units on their lysine residues or on their N-terminus (Breitschopf et al., 1998; Shamu et al., 2001; Shamu et al., 1999; Yoshida et al., 2002). Polyubiquitylation is used to continue retrotranslocation and is needed to dissociate the retrotranslocated protein from the ER and for successful proteasome degradation (Biederer et al., 1997). Monoubiquitylation is insufficient for proper degradation (Shamu et al., 2001; Ward et al., 1995; Yu and Kopito, 1999). Complete retrotranslocation and presentation of misfolded proteins may require ATPase activity (Meyer et al., 2000; Rabinovich et al., 2002; Ye et al., 2001).

After ubiquitylation, glycoproteins in the cytosol are deglycosylated by peptide: N-glycosidase (PNGase) enzymes which convert the amide group of asparagine into an acid group (Hagihara et al., 2007; Parodi, 2000; Romisch and Ali, 1997; Suzuki et al., 1998). This step, although not essential, facilitates degradation (Hagihara et al., 2007). The ubiquitylated and preferably deglycosylated proteins are recognized by the 19S subunits of the proteasome.
through their polyubiquitin chains and are channeled through the barrel-like 20S subunit. This section of the proteasome complex contains the proteolytic sites which will degrade the protein (Figure 1(7)) (Voges et al., 1999).

Not all ERAD substrates are degraded once they reach the cytosol. In fact, the cholera toxin becomes activated once it reaches the cytosol after retrotranslocation (Schmitz et al., 2000). Endogenous proteins can also use ERAD-like retrotranslocation as part of their physiological function. For instance, in order to reach the nucleus, the ligand-bound transmembrane epidermal growth factor (EGF) receptor leaves the cell surface, reaches the ER via internalization and vesicular transport and gets retrotranslocated to the cytosol via the Sec61 complex before it is targeted to the nucleus (Liao and Carpenter, 2007). Clusterin also seems to evade degradation after being retrotranslocated to the cytosol, possibly to reach the nucleus as well (Jones and Jomary, 2002; Nizard et al., 2007). PrP is also able to escape the ERAD pathway (Jodoin et al., 2007; Lin et al., 2008).

1.3 CyPrP

Up to 10% of all synthesized PrP appears in the cytosol after going through the ERAD pathway (Ma and Lindquist, 2001; Yedidia et al., 2001). The main evidence that PrP is retrotranslocated to the cytosol comes from its accumulation in the cytosol after proteasome inhibition (Ma and Lindquist, 2001; Roucou et al., 2003; Yedidia et al., 2001; Zanusso et al., 1999). However, some groups argue that this accumulation is due to proteasome inhibition-induced upregulation of expression of the cytomegalovirus (CMV) promoter, often used to
overexpress PrP (Drisaldi et al., 2003). Nonetheless, retrotranslocated PrP can also be observed in absence of proteasome inhibition (Ma and Lindquist, 2001) and from endogenously expressed PrP in primary neurons (Roucou et al., 2003). Additional confirmation that the observed cytosolic PrP (CyPrP) is derived from the ER is obtained through demonstration of the cleavage of the N-terminal signal peptide through the use of antibodies targeted to this region (Roucou et al., 2003; Wang et al., 2005). Before, evidence came from comparing the size of the putative CyPrP to that of recombinant PrP lacking the two signal peptides (Ma and Lindquist, 2001).

CyPrP may also be produced after improper translocation into the ER so that the nascent chain is directly translated in the cytosol, in which case PrP keeps both its signal sequences (figure 1(8)). This species of PrP is thus known as SP-CyPrP (Rane et al., 2004; Drisaldi, 2003) and is a result of either overexpression or ER stress (Drisaldi et al., 2003; Orsi et al., 2006).

CyPrP ubiquitylation has been observed in several cell lines (Yedidia et al., 2001) but not in cultured human primary neurons (Roucou et al., 2003) nor in the Y145stop PrP mutant-transfected M17 neuroblastoma cells (Jin et al., 2000; Zanusso et al., 1999). This does not completely exclude ubiquitylation as ubiquitin chains may be rapidly removed during the analysis procedure. The observed retrotranslocated CyPrP was mostly devoid of glycan moieties (Ma and Lindquist, 2001), although glycosylation was observed in presence of epoxomicin (Jodoin et al., 2007; Karaivanova and Spiro, 2000). Retrotranslocated PrP is primarily degraded by the proteasome, but when the complex is compromised, cytosolic proteases such as calpain, can take over (Wang et al., 2005; Yadavalli et
al., 2004). Similar to the retrotranslocated form of CyPrP, SP-CyPrP is ubiquitylated and degraded by the proteasome as well (Figure 1) (Fioriti et al., 2005; Rane et al., 2004).

CyPrP interacts with ER and plasma membrane lipids in transfected N2a cells and in CyPrP transgenic mice on a PrP knock-out background (Wang et al., 2006). In cerebellar neurons, CyPrP interacts with the hydrophobic core of these membranes (Wang et al., 2006) while elsewhere in the mouse brain, the interaction between lipids and CyPrP is limited to contact with the hydrophilic surface of these membranes. Interestingly, the cerebellum has also been shown to be the only brain site where CyPrP was shown to be toxic in mice (Ma et al., 2002). This direct membrane interaction may correlate with toxicity.

CyPrP has been shown to be toxic in N2a cells, HEK 293 cells, and in CyPrP transgenic mouse cerebellar neurons (Ma and Lindquist, 2002; Ma et al., 2002). In these models, CyPrP may acquire properties of the disease-associated form of PrP such as partial resistance to proteinase K (PK) cleavage (Ma and Lindquist, 2002) or the formation of aggregates (Grenier et al., 2006; Yedidia et al., 2001). However, one group has observed by immunofluorescence studies that conditional expression of CyPrP produced nuclear PrP (Crozet et al., 2006). Hence, the observed toxicity and aggregation may be due to abnormal presence in the nucleus rather than in the cytosol.

Other groups have also observed PrP aggregates in the cytosol but have found those to be harmless in mouse neuronal cell lines (Kristiansen et al., 2005). The same phenomenon was observed for SP-CyPrP in N2a cells (Fioriti et al., 2005). In addition CyPrP mice on PrP knock-out background were resistant to
misfolded PrP infection (Norstrom et al., 2007). In primary human neuron cultures and in MCF-7 cells, CyPrP was even shown to be beneficial (Jodoin et al., 2007; Lin et al., 2008; Roucou et al., 2003).

CyPrP has been observed in vivo in neurons from the hippocampus, thalamus and somatosensory neocortex of mice (Mironov et al., 2003). Surprisingly, PrP was also found in cytosolic inclusions of pancreatic cells of rat models of type-1 diabetes (Strom et al., 2006). Because the proteasome can be impaired with aging (Gray et al., 2003), CyPrP may very well be more than an artifact enhanced by chemical proteasome inhibitors.

2. PrP structural elements

2.1 Primary structure

PrP structure is similar amongst mammalian species but local interactions and instabilities can differ (Dima and Thirumalai, 2002; Viles et al., 2001).

The 253-residue human PrP contains two well-conserved signal sequences at its N- (amino acids 1-22) and C-termini (232-253) (Figure 2A) (van Rheede et al., 2003). As mentioned previously, the N-terminal signal peptide targets PrP to the ER (Basler et al., 1986) and the C-terminal peptide signals the addition of the GPI anchor. Both are cleaved off during PrP’s transit in the ER (Kretzschmar et al., 1986; Robakis et al., 1986; Rogers et al., 1993). The N-terminal half of PrP also contains a stop-transfer effector (STE) sequence (104-111) and a transmembrane domain (TM) (112-135) required for the aforementioned transmembrane forms of PrP (Lopez et al., 1990; Yost et al., 1990). The STE and
TM regions are among the best-conserved in PrP (van Rheede et al., 2003). In addition, PrP contains in its N-terminal half five octapeptide repeats (ORs, residues 51-91). Similar repeats are found in the yeast prion protein Sup35 (Parham et al., 2001), but not in *Xenopus laevis* PrP (Nunziante et al., 2003). The number and content of the ORs in mammalian species vary since in squirrel PrP, the ORs can lack some of the functionally important histidines (Schatzl et al., 1995; van Rheede et al., 2003). Additional repeats can appear during meiosis in the human *PRNP* gene (Cannella et al., 2007). Embedded in these standard ORs are four other octapeptide repeats that show similarity to the BH2 domain of the anti-apoptotic B cell lymphoma/leukemia 2 (Bcl-2) protein (LeBlanc, 1998). To avoid confusion, this second series of repeats will be referred to as the BH2-like octapeptide repeats (BORs).

### 2.2 Secondary structure

Nuclear magnetic resonance (NMR) and X-ray crystallography experiments show that the N-terminus of folded PrP remains a flexible tail (residues 23-127) while the C-terminal half folds into a globular domain which contains a small two-stranded beta-sheet and three alpha helices (Figure 2B) (Donne et al., 1997; Zahn et al., 2000). The two C-terminal helices are linked by a
Figure 2. PrP primary and secondary structure elements

A. Schematic diagram of the main structural elements of PrP. Abbreviations: SP: ER-targeting signal peptide. BOR: BH2-like octapeptide repeats (residues 56-87 vs. 51-91 for the standard ORs). STE: Stop-transfer effector sequence. TM: transmembrane domain. β1 and β2: beta-strands 1 and 2. α1, α2, α3: alpha-helices 1, 2, and 3. GPI: GPI anchor signal. B. Three-dimensional structure of the PrP globular domain showing the position of the secondary structure elements, the disulfide bond (S-S), the glycosylation sites N181 and N197 and of the polymorphic 129M/V residue (methionine shown). C. Three-dimensional representation of the helix 1-stabilizing interactions between D144 and R148 and between D147 and R151. D. Main elements of the helix 3 “cap” structure (see text for details).
Figure 2.

A. 

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B. 

C. 

D.
disulfide bond (Haire et al., 2004; Riek et al., 1996; Riek et al., 1997; Zahn et al., 2000). The OR region, a part of the N-terminal flexible tail, is thought to adopt a helical conformation as well (Smith et al., 1997). However, other groups say it remains unstructured (Viles et al., 2001). None of the helices of PrP are amphipathic (Dima and Thirumalai, 2002) and interestingly, many amino acid residues of PrP are in unconventional positions in regards to solvent exposure, helix propensities, or bond angles (Dima and Thirumalai, 2002).

2.2.1 Beta-sheet

The beta-sheet is formed from two anti-parallel strands at positions 128-131 and 161-164 (Riek et al., 1996; Riek et al., 1997; Zahn et al., 2000), and thus contains the polymorphic residue at position 129 on the surface of PrP (Figure 2B) (Zahn et al., 2000). Interestingly, in a NMR and circular dichroism (CD) study of a peptide spanning the two strands and helix 1, the residues in the strands have a tendency to become helical (Sharman et al., 1998). CD analyses of peptides can be greatly influenced by salts, especially anions, which tend to be overlooked (Ronga et al., 2006a). In addition, the peaks for alpha-helices overlap with those for beta-sheets (Ollesch et al., 2007). Hence, errors in structure determination are possible with this method and CD results should be supported by another structural determination method like NMR, as above.
2.2.2 Helix 1

Helix 1 (residues 144-154) has a highly charged surface (Zuegg and Gready, 1999), and is made almost entirely of hydrophilic residues (Dima and Thirumalai, 2002; Morrissey and Shakhnovich, 1999). This abundance of hydrophilic residues allows the formation of two internal salt bridges that add to the hydrogen bonds maintaining the helix 1 structure (Figure 2C) (Dima and Thirumalai, 2004; Morrissey and Shakhnovich, 1999). In fact, the double mutant D147A/R151A, which targets the salt bridge linking these residues, destabilizes helix 1 (Dima and Thirumalai, 2004). In sheep PrP, a mutant targeting the equivalent salt bridge destabilizes helix 1 (Rezaei et al., 2002). However, in vitro conversion assays on double mutants targeting the aspartates in the salt bridges suggest these do not contribute much to helix 1 stability (Speare et al., 2003). Hence, it is not clear at this time if the presence of these bridges contribute to helix 1 stability.

The very stability of helix 1 is controversial. Some groups suggest that it is the most stable of the three PrP helices based on high temperature molecular dynamics (MD) simulations involving implicit water (Levy and Becker, 2002). In wild-type PrP, helix 1 is predicted to remain intact during unfolding (Levy and Becker, 2002) and at high temperatures (Colacino et al., 2006). On the other hand, the work of Levy and colleagues found that at low pH, helix 1 was the most mobile of the three (Levy and Becker, 2002) and Viles and colleagues found that this helix was the most flexible (Viles et al., 2001).
CD and NMR analyses show that a PrP-derived peptide containing helix 1 showed high helical propensity and content in various mixtures containing water and the helical content-enhancing agent trifluoroethanol (TFE) (Liu et al., 1999; Sharman et al., 1998; Ziegler et al., 2003). Similarly, infrared spectroscopy and electron microscopy show that helix 1 is the only PrP structural element that does not form fibrils (Jamin et al., 2002). Usually, helices isolated from the rest of the protein are barely stable, making this helix even more unique (Dima and Thirumalai, 2004). But again, similar methods found that a peptide spanning residues 142-166 of PrP adopts a beta-hairpin conformation (Kozin et al., 2001). In water, CD analyses found that the helix 1 peptide adopts a random coil conformation. Addition of TFE assists in the adoption of a helical form but this conformation is sensitive to low pH (Gallo et al., 2005). The debate on helix 1 stability may have been resolved by yet another MD analysis. On its own, the fragment is stable but this stability is lost when helix 1 is part of the full PrP, possibly because of an interaction with R208 in helix 3 (Ji et al., 2005).

2.2.3 Helix 2

On its own, helix 2 (residues 173 to 194) (Zahn et al., 2000) has a high tendency to adopt a beta-sheet structure (Ronga et al., 2006b). This may be due to most of the hydrophobic residues being grouped in the same area of the helix (Dima and Thirumalai, 2002). The region encompassing residues 179 and 191 is predicted to form a beta-sheet. This discrepancy between beta-sheet-predicted and alpha-helix-experimental structure has been correlated with a tendency to form amyloid fibrils (Kallberg et al., 2001). The PrP 178-193 peptide shows similarity
with the amyloid beta peptide (Thompson et al., 2000). The second half of helix 2 is believed not to stay helical for more than a few nanoseconds in MD studies of a peptide overlapping helices 2 and 3 (Dima and Thirumalai, 2004). Yet in a water-TFE mixture, helix 2 has a greater tendency to adopt a helical conformation than helix 1. This tendency is kept in a 50% TFE solution and in 50% ethanol, although in this last condition, helix 2 is less than 10% helical (Gallo et al., 2005). CD analysis of a PrP 173-195 peptide in various conditions shows that helix 2 can easily shift between alpha-helical and beta-strand conformation yet has a slight preference for the helical form in physiological conditions (Tizzano et al., 2005). The small difference between the two states suggests the immediate environment can greatly influence the final conformation of the peptide (Tizzano et al., 2005). A study on the C-terminal domain of PrP lacking helix 1 comes to similar conclusions after the region corresponding to helices 2 and 3 was more disordered in this truncated domain or as an isolated peptide than in the intact globular domain (Eberl and Glockshuber, 2002). In addition, the end of helix 2 (residues 187 to 194) is more disordered and its tendency to remain helical depends on the length of the N-terminal flexible tail (Zahn et al., 2000). Other groups say this instability is not due to the absence of support from the rest of the PrP globular domain because that part of helix 2 has limited contact with the rest of PrP (Dima and Thirumalai, 2004). Nevertheless, helix 2 forms with helix 3 the core of native PrP (Viles et al., 2001), and also of PrP fibrils (Lu et al., 2007) thus the structure of helix 2 in the complete PrP will be influenced by the rest of the protein.
2.2.4 Helix 3

As mentioned previously, helix 3 (residues 200-227) (Zahn et al., 2000) forms the other half of PrP’s rigid core with helix 2 (Viles et al., 2001). This last PrP helix has 5 residues per turn while most helices have only 3 to 4 residues per turn (Dima and Thirumalai, 2002). Helix 3 has a conserved S/T-X-X-D/E cap motif at its N-terminus at residues T199 and D202, surrounded by a hydrophobic staple motif at F198 and V203. In addition to these interactions, a salt bridge connects E200 and K204 (Figure 2D). However, CD comparison of helix 3 peptide mutants affecting these interactions show that only the hydrogen bond cap and salt bridge have a tangible effect on helix 3 stability (Gallo et al., 2005).

In solution, CD and NMR studies have shown that helix 3, but not the other PrP helices, has full structural autonomy (Gallo et al., 2005). As a peptide, helix 3 is able to acquire its helical structure on its own, with the help of the well-conserved capping box and salt bridge (Gallo et al., 2005). However, in water, at a peptide concentration 80mM, the helix 3 peptide is not stable (Gallo et al., 2005). In a solution containing 50% TFE, the helical content of the peptide was almost 50% and reached 38% in a 50% ethanol solution (Gallo et al., 2005). In fact, the NMR structure of this helix 3 peptide in 50% TFE is almost indistinguishable from that of helix 3 in PrP 121-231 (Gallo et al., 2005).

Unlike helix 2, helix 3 has a low tendency to form beta-sheets (Ronga et al., 2006b) and conserves its structure during PrP unfolding induced by low pH (Colacino et al., 2006). However, helix 3 tends to bend between residues 212 and 213 (Zuegg and Gready, 1999) and its C-terminus can easily lose stability (Zahn
et al., 2000). In fact, in the original NMR structure of PrP, done for residues 121-231 of the mouse protein, the portion between residues 220 and 227 was thought to be disordered and not part of an helix (Riek et al., 1996). The instability of helix 3 C-terminus was also demonstrated in MD studies of the peptide containing helices 2 and 3 of PrP described earlier (Dima and Thirumalai, 2004). The stability of the C-terminus of helix 3 can be controlled through interactions with the flexible tail, as for helix 2 (Zahn et al., 2000). In fact, the NMR structure of N-truncated forms of PrP tends to have a shorter helix 3 (James et al., 1997; Zahn et al., 2000).

2.3 Tertiary structure and local interactions

In order to form the globular domain of PrP, secondary structure elements must interact with each other via hydrophobic interactions, salt bridges, hydrogen bonds and even covalent interactions (Pace et al., 1996). However, unlike most proteins, which will have mostly hydrophobic interactions, local interactions in PrP involve an unusually high number of contacts between charged residues and between hydrophobic or polar residues with polar or negatively charged residues. The amount of local contacts between hydrophobic or polar residues and negatively charged residues in PrP is more similar to what is found in beta-sheet-rich proteins despite cellular PrP being mostly alpha-helical (Dima and Thirumalai, 2002).
2.3.1 PrP core

Composition of the stable core of PrP is controversial. Zuegg and Gready believe helices 2 and 3 are the main elements of this core, which are then connected to the more flexible helix 1 and beta-sheet through salt bridges (Zuegg and Gready, 1999). Interestingly, the exclusivity of hydrophilic residues in helix 1 prevents interactions with the hydrophobic core of PrP (Morrissey and Shakhnovich, 1999), which may explain the flexibility of this region (Viles et al., 2001). Others believe the PrP core links residues from all three helices in a series of hydrophobic interactions (Gallo et al., 2005; Haire et al., 2004). In this model, the entire helix 1 is part of the stabilizing core of PrP as shown by energy analyses (Colacino et al., 2006). The loop preceding helix 3 and its N-terminus (residue 194-208) form the stabilizing core of PrP with helix 1 (Colacino et al., 2006).

2.3.2 Local interactions

Helix 1 is almost perpendicular to helix 3, which follows the long axis of PrP in its three-dimensional native conformation (Viles et al., 2001). Several interactions link the two helices including very stable salt bridges (Figure 3A). The most stable salt bridge links E146 and R208 (Zuegg and Gready, 1999). The bridge between R156 and E196 brings helices 1 and 3 closer to each other even though the linked residues are not part of either helix (Zuegg and Gready, 1999). R156 also bonds with D202; but that bridge is only seen in MD simulations involving chloride ions (Figure 3A). Hence, the physiological relevance of this interaction remains to be proven (Zuegg and Gready, 1999). D202 also bonds with Y149 in helix 1 and with Y157 just outside of the helix (Figure 3A). In sheep
PrP, R208 and K204 coordinate a phosphate ion that also interacts with E146, G142 and N143 (Haire et al., 2004). However, salt bridges are not always stabilizing PrP structure. In fact, helix 1 is thought to be less stable in PrP than on its own because of an ionic interaction between its D147 with R208 in helix 3 (Ji et al., 2005).

Helix 2 and the two-stranded beta-sheet are linked by a salt bridge between D178 and R164 but studies involving a mutation affecting D178 have shown that it is not essential to maintain the overall structure of PrP (Gsponer et al., 2001). Thus, it seems the hydrogen bonds linking the two, notably those linking Y162, C179, and T183 together, are more important for stability (Figure 3B) (Riek et al., 1998; Zuegg and Gready, 1999).

As mentioned above, helices 2 and 3 are thought by some groups to form the core of the globular domain of PrP (Zuegg and Gready, 1999). This is supported by the disulfide bond connecting the two (Turk et al., 1988). The importance of that bond has been tested and has given mixed results: one group found that at neutral pH, the PrP 91-231 fragment missing the disulfide bond had essentially the same structure as the same fragment with the bond intact (Jackson et al., 1999), while others found that the disulfide bridge reduction altered the structure of PrP to a beta-sheet-rich form (Ollesch et al., 2007). The residues surrounding the S-S bridge form hydrophobic interactions amongst themselves (Dima and Thirumalai, 2002). Outside of this bond, residues 166 and 220 contribute to bringing the two helices together, but not via an interaction with each other (Calzolai et al., 2000). Interestingly, this two-helix bundle also interacts with the flexible N-terminal tail (Kachel et al., 2006; Li et al., 2000c).
Figure 3. Local interactions implicated in PrP tertiary structure

A. Residues and interactions bringing together helices 1 and 3 of PrP (see text for details). B. Residues and interactions connecting the beta-sheet and helix 2 of PrP (see text for details).
Figure 3.

A.

B.

β-sheet
2.3.3 Scrapie form of PrP

As mentioned earlier, PrP is most studied in the context of the diseases, collectively known as TSEs. Its misfolded scrapie form causes disease various mammalian species. These disorders arise from either inherited mutations, spontaneous misfolding, or transmission of misfolded PrP between individuals (Prusiner, 1998). The scrapie form of PrP (PrPSc) is prone to aggregation and is partially resistant to PK cleavage (McKinley et al., 1983). The PK cleavage site in PrPSc appears to be inside the OR region (Georgieva et al., 2004). What is most striking about this form of PrP is that the difference between it and the normal PrP is at the tertiary structure level, as PrPSc is mostly formed of beta-sheets while normal PrP is mostly alpha-helical (Caughey et al., 1991; Pan et al., 1993). And more importantly, the scrapie form is hypothesized to “convert” normal PrP into the scrapie form and therefore propagate itself (Kocisko et al., 1994; Prusiner, 1991; Prusiner et al., 1990). This conversion is thought to require a hypothetical chaperone designated as “protein X” (Telling et al., 1995).

2.3.4 Disease-associated PrP mutations

Mutations such as those seen in inherited prion diseases may destabilize PrP structure (Liemann and Glockshuber, 1999; Swietnicki et al., 1998) or interfere with the PrP folding pathway (Apetri et al., 2004). Some of these are found in healthy animals hence their link to disease is uncertain (van Rheede et al., 2003).
PrP mutations are generally localized in the folded C-terminus of PrP (Riek et al., 1998). That said, some are localized in the flexible N-terminal tail, namely P102L, P105L and A117V, but their effects on PrP structure are limited (Apetri et al., 2004; Swietnicki et al., 1998).

In the beta-sheet, although not exactly a mutation, the presence of a methionine at position 129 in PrP promotes aggregation by exposing helix 1 to aggregation (Pham et al., 2008).

In helix 2, several mutations are related to disease and to misfolding, which is expected since it is part of the PrP core (Colacino et al., 2006; Zuegg and Gready, 1999). One of the most thoroughly studied PrP mutant is D178N, which affects stability of all three helices in unfolding situations and prevents the PrP folding pathway from being completed (Apetri et al., 2004). Yet, based on MD simulations, D178N does not greatly affect the native helical and beta-sheet content of PrP (Levy and Becker, 2002). Nevertheless, this mutation removes a hydrogen bond with Y128 and a salt bridge with R164 (Figure 3B) (Levy and Becker, 2002) in addition to creating a kink in a helix 2-derived peptide (Ronga et al., 2007). Similar to D178N, the V180I mutation hinders PrP folding (Apetri et al., 2004) but ultimately, this mutant is not predicted to have profound effects on PrP structure since there is a sufficiently large area for the larger side chain (Riek et al., 1998). On the other hand, T183A not only affects PrP structure through the breakage of a hydrogen bond with the beta-sheet (Figure 3B), but the resulting destabilization interferes with both glycosylation at N181 and the addition of the GPI anchor (Kiachopoulos et al., 2005). The result is PrP misfolding into a protease resistant protein (Kiachopoulos et al., 2005).
Although the F198S mutation is not part of helix 3, the phenylalanine residue is required for stabilizing the N-terminus of the helix (Figure 2D) (Gallo et al., 2005; Liemann and Glockshuber, 1999; Vanik and Surewicz, 2002). Hence, this mutation interferes with the folding and glycosylation of PrP as well as with the addition of the GPI anchor (Apetri et al., 2004; Kiachopoulos et al., 2005) and again, alters PrP structure to a misfolded, protease-resistant form (Kiachopoulos et al., 2005; Vanik and Surewicz, 2002).

Similar to those in helix 2, helix 3 mutations tend to have a greater impact on PrP structure because helix 3 is part of the PrP core. The E200K mutant affects helix 3 capping by disrupting the salt bridge it forms with K204 (Figure 2D) (Gallo et al., 2005) and can interfere with folding as for previously discussed mutations (Apetri et al., 2004). Yet in the final PrP structure, the effects on PrP stability are mild (Apetri et al., 2004; Riek et al., 1998), despite possible changes to the surface electrostatic potential (Zhang et al., 2000). On the other hand, the M205R and M205S mutations quickly and severely affect PrP structure through the disturbance of the interactions between helices 1 and 3 (Figure 3A) (Hirschberger et al., 2006). The R208H mutation does interfere with the salt bridge it forms with residues in helix 1, but mutations on the residues with which R208 forms salt bridges show that the impact of R208H on PrP structure is due to general effects on structure rather than specific disturbances of these bridges (Bamdad and Naderi-Manesh, 2007). The V210I mutant does not affect the native structure of PrP per se; V210I rather stabilizes misfolded forms of PrP (Thompson et al., 2001). Q217R interferes with its own folding by staying associated to the Bip chaperone for an unusually long period of time (Jin et al., 2000) and
remaining in the ER with an uncleaved GPI anchor signal (Singh et al., 1997). The end result is an unstable protein with re-arranged hydrogen bonds (Liemann and Glockshuber, 1999; Wong et al., 2000).

2.4 Quaternary structure

The cellular form of PrP is thought to exist as a monomer. However, once crystallized, PrP forms a dimer. In this form of PrP, the C-terminal part of helix 2 (189-198) from each monomer is shown to form a beta-sheet linking the two monomers while the rest of the helix is linked to helix 3 from the second monomer by a disulfide bond (Knaus et al., 2001). Whether this dimer form of PrP is more than an artifact of crystallization remains to be seen, especially since a monomeric form of PrP has been crystallized in sheep (Haire et al., 2004). There is consensus about the aggregation of the disease-associated form of PrP (Yedidia et al., 2001).

3. PrP Family

3.1 Doppel

Two proteins homologous to PrP have been identified thus far. The first one, Downstream prion protein-like (Doppel), is located downstream of the PrP-encoding \textit{PRNP} gene as the name implies (Li et al., 2000a; Moore et al., 1999). Unlike PrP, Doppel has little to no CNS expression (Moore et al., 1999; Silverman et al., 2000). If anything, it is involved in the formation of the blood-brain barrier in early development (Li et al., 2000b) but in adult organisms Doppel
is associated with neuronal cell death (Moore et al., 1999). Doppel is rather more highly expressed in lymphoid tissues, heart, and testes (Li et al., 2000b; Moore et al., 1999; Paltrinieri et al., 2004; Silverman et al., 2000). It is in this last organ that Doppel mediates its main role in sperm development (Behrens et al., 2002). In terms of structure, Doppel shares 24% sequence homology with PrP (Moore et al., 1999). Nevertheless, Doppel still has many structural elements that are similar to those of PrP. It has the N- and C-terminal signal sequences that mediate its entry into the ER (Li et al., 2000a; Moore et al., 1999) and the addition of a GPI anchor (Moore et al., 1999; Silverman et al., 2000). As a result, Doppel is also a GPI-anchored surface glycoprotein (Li et al., 2000a; Moore et al., 1999; Silverman et al., 2000). Its three-dimensional structure is also very similar to PrP with a flexible tail and a globular domain with three alpha-helices and a small beta-sheet (Lührs et al., 2003; Mo et al., 2001). However Doppel does differ from PrP in some areas. One of the two glycosylation sites of Doppel is localized between the first and second helices instead of between the second and third in PrP (Li et al., 2000a; Moore et al., 1999; Silverman et al., 2000). Doppel also has a second disulfide bond, (Li et al., 2000a; Moore et al., 1999) and lacks the OR domain (Li et al., 2000a; Moore et al., 1999). Furthermore, the beta-sheet is in the reverse orientation compared to PrP (Lührs et al., 2003; Mo et al., 2001).

3.2 Shadoo

More recently, a second PrP like-protein, Shadoo, has been identified (Premzl et al., 2003). Unlike PrP and Doppel, the SPRN gene is located on chromosome 10 (Premzl et al., 2003). Shadoo has been detected in both fish and
mammals in brain, retina and embryonic tissue (Premzl et al., 2003). Shadoo function seems to overlap with that of PrP as it is able to protect against Doppel and N-truncated PrP toxicity (Watts et al., 2007). Although Shadoo does not have a defined secondary structure (Watts and Westaway, 2007), its primary sequence has elements similar to those of PrP. Shadoo contains the signal sequences that allow it to be synthesized into a surface GPI-anchored glycoprotein (Premzl et al., 2003), as are PrP and Doppel. The N-terminus of Shadoo bears strong homology to PrP with a similar hydrophobic region and a series of repeats though instead octapeptide repeats, Shadoo has a stretch of arginine-rich tetrapeptide repeats (Premzl et al., 2003). Differences with PrP are in the size of the protein as Shadoo is shorter by over 100 residues, the absence of disulfide bonds and the presence of only one glycosylation site instead of two (Premzl et al., 2003).

4. PrP Function

4.1 Knock-out models

The very necessity of PrP has been questioned by the apparently normal phenotype of the Zurich and Edinburg lines of PrP-null mice up to the age of seven months (Bueler et al., 1992; Manson et al., 1994). On the other hand, after 70 weeks, the Nagasaki and Rcm0 strains of PrP knock-out mice developed ataxia with cerebellar atrophy and Purkinje cell loss (Moore et al., 1999; Sakaguchi et al., 1996). This phenotype was later found to be due to the inappropriate expression of the PrP-like protein Doppel (Moore et al., 1999), which causes neurodegeneration in absence of PrP (Moore et al., 1999; Moore et al., 2001;
Rossi et al., 2001). In fact, crossing these mice with PrP overexpressing-mice was sufficient to rescue the ataxic phenotype (Nishida et al., 1999). Nevertheless, upon closer examination, removal of the PRNP gene did have effects in mice, notably, altered circadian rhythms and sleep patterns (Tobler et al., 1997; Tobler et al., 1996). In addition, these mice were more sensitive to toxicity induced by Doppel or N-truncated PrP, induced seizures, and ischemia than wild-type mice (Mitteregger et al., 2007; Moore et al., 2001; Shmerling et al., 1998; Walz et al., 1999). Hence, initial studies of knock-out models of PrP were not sufficient to clarify its physiological role.

4.2 Functions attributed to PrP

PrP is linked to many roles both within the nervous system and elsewhere in the body. PrP has been implicated in the activation and proliferation of T-cells in response to mitogens as both phenomena were enhanced in PrP-expressing cells compared to those lacking PrP (Bainbridge and Walker, 2005; Cashman et al., 1990). T-cells activation and proliferation were inhibited by the addition of anti-PrP antibodies (Li et al., 2001b). Similar effects were observed for PrP in antigen-presenting cells (APC) with PrP being upregulated during their activation and maturation (Martinez del Hoyo et al., 2006). Interestingly, PrP was even found at the interface between dendritic cells, one type of APC, and T-cells (Linden et al., 2008). Although PrP is implicated in the interactions between these two cell types, the nature of this role remains to be determined.

PrP is thought to play a role at various levels in the nervous system starting with neuronal development. Higher PrP levels facilitate in vitro
differentiation. In the dentate gyrus region of the hippocampus, which is capable of neurogenesis in adult animals, PrP-overexpressing mice have a higher number of proliferating cells than wild-type or PrP-null mice (Steele et al., 2006). Factors other than PrP must influence neuronal development given the constant final number of neuronal cells regardless of PrP expression levels (Steele et al., 2006). From neural development, PrP has also been implicated in synaptic function. First clues to such a role came from the localization of PrP at synapses shown by immunogold techniques (Fournier et al., 2000; Fournier et al., 1995). However, others have observed an even distribution of PrP on the cell surface (Mironov et al., 2003). Nevertheless, functional evidence also supports a role for PrP in synapses as brain slices from PrP-null mice displayed abnormal gamma-amino butyric acid (GABA)-mediated inhibitory post-synaptic potentials and currents (Collinge et al., 1994). Although these effects were not reproduced in younger mice, (Lledo et al., 1996), a conditional PrP-null mouse also displayed altered neurophysiology (Mallucci et al., 2002) and high-level PrP overexpression was able to rescue the phenotype (Whittington et al., 1995).

This role in synaptic function may specifically affect memory. Hippocampal brain slices from PrP null-mice presented abnormal long-term potentiation (LTP) (Collinge et al., 1994), a mechanism thought to underlie memory (Bliss and Collingridge, 1993). Beyond the molecular level, altering PrP has effects on behavior. Disruption of PrP interaction with stress-inducible protein 1 (ST1) impairs both short and long-term inhibitory avoidance learning in aging rats (Coitinho et al., 2007; Coitinho et al., 2003). Spatial memory is also affected in PrP-null mice and PrP overexpression in neurons rescues this deficit (Criado et
Strikingly, the influence of PrP on memory is even seen in human subjects as individuals homozygous for the 129V allele performed more poorly on a long-term word recall task than heterozygous or 129M homozygous individuals (Papassotiropoulos et al., 2005). This link to memory could even have implications in Alzheimer’s disease since PrP has been involved in regulation of beta-secretase cleavage of the amyloid precursor protein. Overexpression of PrP reduces cleavage and amyloid beta peptide production and PrP-null mice have higher levels of amyloid beta peptide, one hallmark of the disease (Parkin et al., 2007).

4.3 Protective functions of PrP

PrP has been shown to protect neurons from a variety of insults. For instance PrP-null hippocampal cells are more sensitive to serum deprivation than wild-type cells (Kuwahara et al., 1999). This sensitivity is rescued by overexpression of either PrP or Bcl-2 (Kuwahara et al., 1999). *In vivo*, PrP is upregulated in the hours following ischemic injury induction in mice (Weise et al., 2004) and accumulates in the penumbra of human and rodent brains after injury (McLennan et al., 2004). Similarly, PrP-null mice displayed a larger infarct area following ischemia than wild-type mice (McLennan et al., 2004). In addition to protecting against the damage due to the ischemia, PrP may also protect against the damage associated with reperfusion, that is, oxidative stress (Christophe and Nicolas, 2006). PrP’s anti-oxidative stress role seems related to its ability to bind metal ions in its OR region and outside (Brown et al., 2004; Brown et al., 1997a; Lehmann, 2002). PrP overexpression rescues PrP-null cells against oxidative
stress and this rescue is accompanied by an increase in superoxide dismutase (SOD) activity, although not necessarily its own (Sakudo et al., 2003). Interestingly, in order to protect against oxidative stress, one group has proposed that PrP must be cleaved by reactive oxygen species at a site located after the octapeptide repeats (Watt et al., 2005). Therefore, PrP is thought to either regulate (SOD) activity (Brown and Besinger, 1998; Brown et al., 1999; Jones et al., 2005; Sakudo et al., 2005a) or act as a buffer for metal ions (Lehmann, 2002).

PrP is also able to protect against Doppel overexpression (Nishida et al., 1999). Similarly, PrP protects against the N-truncated version of itself, which resembles Doppel. In vivo, PrP reverses the ataxic phenotype of the Shmerling mouse, which over expresses this truncated form of PrP on a PrP-null background, and rescues associated Purkinje cell loss (Flechsig et al., 2003). Interestingly, PrP can even protect against the neonatal lethality in mice expressing PrP without residues 105 to 125 (Westergard et al.). Therefore, PrP can protect against the toxic effects of its homolog and of modified versions of itself.

PrP’s protective role has implication in cancer. Tumor necrosis factor-alpha (TNF-alpha)-resistant breast carcinoma MCF-7 cells have 17 times the levels of PrP of TNF-alpha-sensitive MCF-7 cells. Once PrP is overexpressed in the sensitive cells, they too become resistant to TNF-alpha (Diarra-Mehrpour et al., 2004). Similarly, high levels of PrP correlate with resistance to TNF-related apoptosis-inducing ligand (TRAIL), a trigger of apoptotic cell death (Meslin et al., 2007b). Moreover, PrP even protects against cell death mediated by anti-cancer drugs. It is upregulated in adriamycin-resistant gastric cancer cell lines.
compared to sensitive lines and as before, PrP upregulation confers resistance to sensitive lines and ribonucleic acid interference (RNAi) downregulation of PrP in the resistant line rendered it adriamycin-sensitive (Du et al., 2005). In human breast tumors, those showing high PrP expression were resistant to post-operation chemotherapy (Meslin et al., 2007a).

5. PrP Anti-Bax function

Interestingly, many of the insults against which PrP protects involve Bcl-2-associated X protein (Bax)-mediated apoptosis. Serum deprivation accelerates Bax conformational change (Roucou et al., 2005), while Bax and Bcl-2 homologous antagonist/killer (Bak) are required to mediate cell death caused by calcium-dependent stimuli such as oxidative stress (Scorrano et al., 2003). Seven-day old Bax knockout mice presented less neuronal cell loss compared to wild-type mice after induction of cerebral ischemia (Gibson et al., 2001). PrP null mice from the Nagasaki strain, which express Doppel in the brain, had less Purkinje cell loss if they were also deficient for Bax (Heitz et al., 2007). These results contradict work in PrP-null mice carrying a Doppel transgene which suggested that such cell loss may not even occur by apoptosis (Dong et al., 2007).

5.1 Apoptosis overview

Apoptosis (Kerr et al., 1972) is a tightly regulated cell death program essential in development, in normal tissue homeostasis and in defense against various cellular insults (Hengartner and Horvitz, 1994; Kerr et al., 1972). This
pathway is conserved throughout evolution to as far as the nematode worm *Caenorhabditis elegans*. This study model allowed the establishment of a rudimentary pathway for apoptosis. The main players in *C. elegans* apoptosis are homologous to various families of mammalian apoptotic regulators (Hengartner and Horvitz, 1994).

**5.1.1 Pathways**

Mammalian apoptosis can generally be triggered either by ligand binding on cell surface “death receptors” or by cellular stress or insults (van Delft and Huang, 2006).

The former trigger leads to the extrinsic pathway initiated by (TNF) superfamily ligand binding to death receptors which, via adaptors, turn on initiator caspases 8 and 10 (Chinnaiyan et al., 1995; Cohen, 1997; Hsu et al., 1995; Kischkel et al., 1995; Martin et al., 1998). Caspase 8 can then cleave and turn on effector caspases 3, 6, and 7 (Danial and Korsmeyer, 2004; Scaffidi et al., 1998). These activated caspases inactivate anti-apoptotic proteins and activate pro-apoptotic proteins (Enari et al., 1998; Liu et al., 1997; Rudel and Bokoch, 1997; Sakahira et al., 1998) in addition to cleaving structural proteins, resulting in cytoskeleton disassembly (Nicholson, 1999). These pro-apoptotic proteins cause blebbing (Rudel and Bokoch, 1997), and DNA fragmentation (Enari et al., 1998; Liu et al., 1997; Sakahira et al., 1998), two morphological hallmarks of apoptosis.

Caspase 8 can also activate BH3 interacting domain death agonist (Bid) (Luo et al., 1998; Wang et al., 1996), a member of the Bcl-2 (Tsujimoto et al., 1984) family of proteins that translocates to the mitochondrion (Luo et al., 1998)
and allows cross-talk with the intrinsic pathway of apoptosis (Li et al., 1998; Luo et al., 1998).

The intrinsic pathway is triggered by deficiencies in nutrients or growth factors, DNA damage and pharmacological insults such as the kinase inhibitor staurosporine (Danial and Korsmeyer, 2004; Kabir et al., 2002). The main checkpoint for this signaling system takes place at the mitochondrion. A group of Bcl-2 family proteins called the “BH3-only” proteins, of which Bid is a member, act as sentinels for cell stress (van Delft and Huang, 2006). Once activated, they release pro-apoptotic Bcl-2 family members Bax and Bak (Chittenden et al., 1995b; Farrow et al., 1995; Kiefer et al., 1995) from the inhibition of their pro-survival homologs (Hinds and Day, 2005). This process occurs via interactions through their Bcl-2 homology (BH) domains (Chittenden et al., 1995a; Yin et al., 1994). Bax and Bak increase the permeability of the mitochondrial membrane, possibly through their pore-forming ability, to allow the release of apoptosis effectors such as cytochrome c (Shimizu et al., 1999). Cytochrome c joins apoptotic protease activating factor-1 (Apaf-1) (Liu et al., 1996; Zou et al., 1997) and pro-caspase 9 to form the apoptosome (Li et al., 1997; Srinivasula et al., 1998). The completed complex can then activate caspase 9 which triggers the caspase cascade and turns on effector caspases (Slee et al., 1999; Srinivasula et al., 1998) to cause blebbing and DNA fragmentation as for the extrinsic pathway.

This model of apoptosis is not always followed to the letter; various external regulators allow bypass of certain steps and cross-talk between pathways. As a result, apoptotic pathways and morphologies may vary enormously between
cell types (Li et al., 2001a; Scaffidi et al., 1998; Susin et al., 2000; Yu et al., 2002).

5.1.2 Bax Overview

Bax is a pro-apoptotic Bcl-2 family protein that carries several BH domains (Oltval et al., 1993; Yin et al., 1994). In the absence of apoptotic stimuli, inactive Bax is either in the cytosol or loosely attached to mitochondria (Suzuki et al., 2000).

Once activated, Bax undergoes a conformational change that exposes its N- and C-termini and allows its integration into the mitochondrial membrane (Suzuki et al., 2000). Once there, Bax can oligomerize with itself (Roucou et al., 2002) or with other Bcl-2 homologs (Sedlak et al., 1995; Suzuki et al., 2000; Yin et al., 1994) to alter the mitochondrial membrane permeability to induce the release of cytochrome c and other pro-apoptotic proteins (Jurgensmeier et al., 1998). The effect of Bax on mitochondrial membrane permeability and cytochrome c release is mediated in conjunction with Bak (Lindsten et al., 2000).

Bax has many inhibitors within the Bcl-2 family, namely Bcl-2 itself (Yin et al., 1994) and B cell lymphoma/leukemia extra-long protein (Bcl-xL) (Yang et al., 1995), but non-Bcl-2 proteins such as Bax inhibitor 1 (BI-1), Bifunctional apoptosis regulator (BAR), clusterin, αA and αB crystallins, humanin, heat shock protein (Hsp) 70, Ku70, and unphosphorylated 14-3-3 protein, can also block Bax-mediated apoptosis. Most of these directly interact with Bax in the cytosol (Gotoh et al., 2004; Guo et al., 2003; Mao et al., 2004; Sawada et al., 2003; Tsuruta et al., 2004) and prevent its translocation to the mitochondrion (Guo et al., 2004).
2003; Mao et al., 2004; Tsuruta et al., 2004; Xu and Reed, 1998; Yang et al., 1995). One of the more unexpected Bax inhibitor is PrP (Bounhar et al., 2006; Bounhar et al., 2001; Gains et al., 2006; Jodoin et al., 2007; Li and Harris, 2005; Roucou et al., 2005; Roucou et al., 2003).

5.2 Models of PrP anti-Bax function

PrP’s specific anti-Bax function has been shown in several model organisms starting with human primary neurons (Bounhar et al., 2001). The paradigm used to induce Bax specific apoptosis was overexpression of Bax since this alone can trigger the conformational change that initiates Bax activation (Roucou et al., 2005). In this system, overexpression of PrP was able to abolish Bax-triggered cell death (Bounhar et al., 2001). PrP protection was shown not to be an artifact of overexpression as downregulation of endogenous PrP by an anti-sense construct increased Bax-mediated cell death (Bounhar et al., 2001).

Overexpressed PrP is also able to protect the NT2 human neuronal cell line (Roucou et al., 2004) as well as the breast carcinoma MCF-7 cell line (Roucou et al., 2004; Roucou et al., 2005) against Bax. In the budding yeast *Saccharomyces cerevisiae*, PrP protects not only against Bax-mediated apoptosis but also against Bax-mediated growth arrest (Bounhar et al., 2006). An earlier yeast study observed a similar effect on growth arrest but the authors interpreted this effect as protection against Bax-mediated cell death (Li and Harris, 2005). Nevertheless, PrP’s effect on growth arrest may not be completely irrelevant to cell death since neurons attempt to re-integrate the cell cycle before dying (Bounhar et al., 2006). *In vivo*, PrP can protect only certain populations of
neurons against ethanol-induced death, a different model of Bax activation (Gains et al., 2006). In this model, the activation of pro-apoptotic factors other than Bax was proposed as a reason for the limited anti-Bax effect of PrP in mice (Gains et al., 2006). However, the anti-Bax function of PrP is cell type-specific as mouse neuroblastoma N2a cells, neuronal SK-N-SH cells, NE(2)-M17 cells, and embryonic kidney HEK293 cells are not protected by PrP against Bax-mediated cell death (Roucou et al., 2004; Roucou et al., 2003).

PrP specifically inhibits Bax-mediated cell death as it cannot protect against other pro-apoptotic Bcl-2 proteins such as Bak or truncated Bid (Roucou et al., 2005). In addition, PrP cannot protect against other apoptotic inducers such as staurosporine or thapsigargin because these insults activate Bak or other pro-apoptotic pathways that induce cell death. Yet, PrP could still prevent Bax activation in these models (Roucou et al., 2005).

5.3 Mechanism of PrP anti-Bax function

The details of the mechanism by which PrP protects against Bax-mediated apoptosis remain a mystery. It is known that PrP prevents the conformational change that triggers Bax activation (Roucou et al., 2005). This active conformation can be detected by the 6A7 anti-Bax antibody. In response to serum deprivation, 6A7-positive Bax was found in PrP-null cells at an earlier time than in wild-type cells. Less Bax could be immunoprecipitated with this antibody in cells transfected with both PrP and Bax (Roucou et al., 2005). Furthermore, in MCF-7 cells, PrP prevents translocation of activated Bax to the mitochondria (Roucou et al., 2005). In neurons, inactive Bax can be loosely attached to the
mitochondrial membrane. Because of the mitochondrial location of inactive Bax, prevention of Bax translocation is difficult to observe in human neurons (Roucou et al., 2005).

PrP’s impact on Bax activation occurs most likely without direct contact between the two proteins. Early evidence was circumstantial: Bax is cytosolic (Suzuki et al., 2000) while most of PrP is at the cell surface or even secreted into the extracellular space (Oesch et al., 1985). In fact, earlier studies using BFA and monensin, two agents that block the secretory pathway, found that PrP had to go through the Golgi in order to protect against Bax (Bounhar et al., 2001). During serum deprivation, PrP remains localized to microsomes while Bax is transiting from the cytosol to the mitochondrial membrane such that a direct interaction is unlikely (Roucou et al., 2005). However, PrP can be detected in the cytosol, if proteasome inhibitors are used, as mentioned earlier (Ma and Lindquist, 2001; Roucou et al., 2003; Yedidia et al., 2001; Zanusso et al., 1999). Therefore, an interaction between PrP and Bax could happen. However, neither the yeast-two hybrid system (Kurschner and Morgan, 1995; Kurschner and Morgan, 1996) nor co-immunoprecipitation of in vitro translated, E. coli-purified or brain-purified proteins has shown direct interaction between PrP and Bax (Lin et al., 2008). Therefore, PrP must have one or more intermediates to execute its anti-Bax function.

The nature of such an intermediate remains elusive. This protein could be a member of the Bcl-2 protein family, perhaps even Bcl-2 itself since yeast-two-hybrid studies have shown that PrP and Bcl-2 can interact (Kurschner and Morgan, 1995; Kurschner and Morgan, 1996). However, interaction between PrP
and Bcl-2 was not observed with co-immunoprecipitation of in vitro-translated, E. coli-, or brain-purified proteins (Lin et al., 2008). Consistent with many other reports, interaction between Bax and Bcl-2 was not observed in this study either (Lin et al., 2008), such that Bcl-2 is not a direct link between PrP and Bax. Furthermore, PrP protects against Bax in absence of any Bcl-2 proteins since it protected against Bax-mediated cell death in yeast, which are devoid of members of the Bcl-2 protein family (Bounhar et al., 2006). Potential non-Bcl-2-related intermediates for PrP anti-Bax function are the identified PrP binding partners also implicated in cell death pathways. Notably, STI-1 interaction with PrP has been shown to mediate neuroprotective signals (Zanata et al., 2002) and phosphatidylinositol 3-kinase (PI3K) is recruited by cellular PrP to transduce cell survival signals (Vassallo et al., 2005).

However, PrP protection against Bax in absence of Bcl-2 proteins does not exclude their involvement in PrP’s anti-Bax function in mammalian cells. Bcl-2 proteins may be one of several intermediates between PrP and Bax since PrP downregulation in MCF-7 cells also downregulates Bcl-2, which allows Bax translocation to the mitochondria (Meslin et al., 2007b). It may very well be that PrP can activate parallel pathways to inhibit Bax activation.

It is also possible that PrP triggers a signaling pathway that leads to decreased Bax expression. By using signaling pathway inhibitors in cerebellar granule neurons, in PrP-null neuronal cells, and in gastric cancer cell lines, several groups have observed that PrP expression correlates with decreased Bax expression (Chen et al., 2003; Du et al., 2005; Kim et al., 2004). The pathway may involve non-receptor tyrosine kinases of the Src family and PI3 kinases, but
not the MAP kinase pathway (Chen et al., 2003). However, Bax expression levels alone cannot be directly related to cell death, since Bax must first be activated (Suzuki et al., 2000). Furthermore, since PrP has been shown to prevent the first step of this activation pathway, Bax conformational change (Roucou et al., 2005), the effects of PrP on Bax expression are most likely secondary. Thus, PrP and the yet unidentified intermediate must work upstream of Bax activation since PrP prevents the conformation changes that triggers Bax activation (Roucou et al., 2005).

Downstream of Bax activation, PrP also prevents cytochrome c release and caspase activation in human neurons, MCF-7 cells and mouse hippocampal cell lines (Kim et al., 2004; Roucou et al., 2005). However, PrP cannot prevent cytochrome c release from mitochondria in a cell-free assay nor can it prevent apoptosis once effector caspases are activated (Roucou et al., 2005).

5.4 Requirements for PrP anti-Bax function

Given the strong structure-function relationship in biology, knowledge of the form and structural elements of PrP required for its anti-Bax function are vital to precisely define the mechanism by which PrP inhibits Bax-mediated cell death.

5.4.1 Topology and localization requirements: the predominant role of CyPrP

As mentioned earlier, PrP is able to adopt several membrane topologies and localizations since it can exist as a transmembrane protein with either its N- or C-terminus facing the ER lumen, as a secreted protein with or without a GPI anchor,
or as a cytosolic protein (Hegde et al., 1998a; Hegde et al., 1998b; Kim and Hegde, 2002; Kim et al., 2001; Ma and Lindquist, 2001; Roucou et al., 2003; Yedidia et al., 2001; Yost et al., 1990; Zanusso et al., 1999). Of these, CyPrP is the predominant anti-Bax form of PrP (Lin et al., 2008). CyPrP is able to protect human neurons against Bax to a similar extent as wild-type PrP (Roucou et al., 2003). Since the human PrP signal peptide does not efficiently mediate PrP translocation into the yeast ER ((Ma and Lindquist, 1999), wild-type human PrP expressed in yeast is mostly cytosolic. This cytosolic form of PrP is able to protect yeast cells against Bax (Bounhar et al., 2006). However, CyPrP is unable to save yeast from Bax-mediated growth arrest (Bounhar et al., 2006; Li and Harris, 2005). Thus, CyPrP is able to protect cells against Bax-mediated cell death.

Subcellular fractionation experiments have shown that decreased CyPrP correlates with loss of anti-Bax function in transmembrane forms of PrP (Lin et al., 2008). A similar link between function and presence in the cytosol has been made for Creutzfeldt-Jakob disease (CJD)-associated familial mutants of PrP since they are not capable of producing CyPrP either (Jodoin et al., 2007). These loss-of-function mutants are thus unable to go through the ERAD pathway and reach the cell surface as misfolded and PK resistant proteins (Jodoin et al., 2007). Lack of CyPrP in all regions of the mouse brain was even put forward as a reason why PrP anti-Bax function was not significant in ethanol-injected mice (Gains et al., 2006; Mironov et al., 2003).

Furthermore, co-expression of wild-type CyPrP or CyPrP with the CJD-associated or topological loss-of-function mutants with their full-length counterpart rescues the anti-Bax function in both human neurons and MCF-7 cells.
Co-expression of wild-type full-length PrP with these mutants is unable to rescue the function (Jodoin et al., 2007). These results confirm that PrP needs to be in the cytosol in order to protect against Bax.

Secreted PrP can also protect against Bax-mediated cell death, but to a lesser extent. Presence or absence of the GPI anchor does not correlate with loss of function in CJD mutants (Jodoin et al., 2007) and constructs encoding secreted PrP lacking the GPI anchor signal (PrPΔGPI) are still able to protect against Bax mediated cell death in human neurons, MCF-7 cells and yeast cells (Bounhar et al., 2006; Bounhar et al., 2001; Lin et al., 2008). Yet, only low levels of protection were observed from media collected from PrPΔGPI-transfected cells (Lin et al., 2008). In addition, presence of secreted PrP does not correlate with anti-Bax function since PrP mutations that favor transmembrane forms of PrP lose the anti-Bax function despite production of secreted PrP (Lin et al., 2008). These results imply that secreted PrP has a limited anti-Bax function, possibly through signaling at a surface receptor rather than signaling from GPI-anchored cell surface PrP (Bounhar et al., 2006; Lin et al., 2008). Nevertheless, secreted PrP protection against Bax plays a minor role compared to CyPrP since cytosolic location correlates with anti-Bax function.

5.4.2 Structure requirements

An extensive study of familial CJD-associated PrP mutants has found no correlation between loss of function and polarity changes, position in the protein, cytotoxicity, protein expression, detergent solubility, PK resistance, or glycosylation (Jodoin et al., 2007). Nevertheless, regions of PrP have been linked
to protection. For instance, the OR region has been linked to protection against oxidative stress (Brown et al., 1997b; Brown et al., 1999) and their removal in hippocampal cells inhibits SOD activity and promotes apoptosis (Sakudo et al., 2005b). In the context of protection against Bax, removal of the BOR domain abolished anti-Bax function in human primary neurons (Bounhar et al., 2001). In yeast cells, OR removal had no effect against Bax-mediated growth arrest, but addition of 9 extra ORs did inhibit growth arrest (Li and Harris, 2005). Furthermore, the BOR region bears similarity to the BH2 domain of Bcl-2, this region is required for Bcl-2 to inhibit Bax activation (LeBlanc, 1998; Yin et al., 1994). Hence, these repeats’ role in anti-Bax function should be investigated further.

Another region of interest is the C-terminal domain of PrP. Even though CJD-associated mutations in this domain only decrease the amount of CyPrP, the main anti-Bax form of PrP (Jodoin et al., 2007), different sites in the PrP globular domain may be directly involved in protection. In fact, the polymorphic site at position 129 of PrP, in the first beta-strand, has an effect on the anti-Bax function that goes beyond retrotranslocation. All CJD mutations associated with a methionine at position 129 lose the anti-Bax function but in wild-type PrP, the presence of methionine has no effect on the anti-Bax function, or retrotranslocation (Jodoin et al., 2007 661). As a result, examination of the involvement of the C-terminal domain in CyPrP can clarify if the involvement of that globular domain is in retrotranslocation or directly in PrP’s anti-Bax function.
6. Deleterious effects

Despite the body of evidence supporting a neuroprotective function, conflicting results show PrP inducing cell death either via its cytosolic form (Ma and Lindquist, 2002; Ma et al., 2002) or via interactions with Bcl-2 (Rambold et al., 2006), the pro-apoptotic protein neurotrophin receptor interacting MAGE homolog (NRAGE) (Bragason and Palsdottir, 2005), or cross-linking antibodies (Solfosori et al., 2004). PrP has even been shown to enhance staurosporine-mediated apoptosis (Paitel et al., 2003). These effects may underline different outcomes of PrP expression in different systems or the possible role of PrP as an apoptosis checkpoint to trigger its inhibition or its activation depending on the stimulus and the cell type.

7. Research hypothesis and objectives

The aim of the present study is to identify the structural elements of PrP necessary for its anti-Bax function. More precisely, we want to know if the BOR and C-terminal domains are involved in the anti-Bax function and the nature of this involvement. We hypothesize that both domains influence PrP anti-Bax function. The BOR domain may act through collaboration between the repeats, similar to what is seen in copper binding (Viles et al., 1999); or in a manner similar to that of the BH2 domain given the resemblance between both domains (LeBlanc, 1998). The C-terminal domain may harbor an active site because its defined three-dimensional structure offers more possibilities for interaction sites.
To verify these hypotheses, we will assay the toxicity and protective function of PrP mutants targeting both regions in MCF-7 cells and in human primary neurons.
CHAPTER 2: HELIX 3 IS NECESSARY AND SUFFICIENT FOR PRION PROTEIN’S ANTI-BAX FUNCTION.

Chapter Preface

This chapter covers the work I did to identify the PrP regions involved in the anti-Bax function. The findings were submitted as a manuscript to the Journal of Biological Chemistry under the following title:


Not included in the manuscript is the data for the expression of the helix 3 peptide. The related data can be found in appendix 1.

Contribution of authors

The majority of the work presented in this chapter was done by me. Dr. Julie Jodoin helped with experimental design, performed the Gene Gun-mediated transfections for the cell death assays (Figures 4C; 5A; 7A and E; 8B and 9B) and the subcellular fractionation procedure (Figure 4D). The manuscript was co-written by all the authors.

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**Introduction**

While the role of prion protein (PrP) in transmissible prion diseases is highly investigated, less is known on the physiological function of the normal cellular PrP. PrP is a highly expressed glycoprotein in brain and peripheral tissues (Prusiner et al., 1998). Most of the PrP is secreted and accumulates on the cell surface as a glycosylphosphatidylinositol (GPI)-anchored protein. However, it was discovered that some PrP is also retrotranslocated in the cytosol where it accumulates in a subset of neurons in the hippocampus, neocortex, and thalamus (Ma and Lindquist, 2001; Mironov et al., 2003; Roucou et al., 2003; Yedidia et al., 2001; Zanusso et al., 1999). PrP promotes neuroprotection *in vivo* and *in vitro*. PrP null mice are more susceptible to ischemia, Doppel and N-terminally truncated PrP-induced cytotoxicity (Mitteregger et al., 2007; Moore et al., 2001; Shmerling et al., 1998). Furthermore, PrP null mice are more susceptible to axotomy-induced cell death and kainic acid-induced seizures (Coulpier et al., 2006; Walz et al., 1999). *In vitro*, PrP protects against oxidative stress (Brown et al., 1997b; Brown et al., 1999), serum deprivation (Kuwahara et al., 1999), and anisomycin-induced cell death (Chiarini et al., 2002; Zanata et al., 2002). PrP renders human breast carcinoma MCF-7 cells resistant to tumor necrosis factor α (Diarra-Mehrpour et al., 2004). Down-regulation of PrP increases sensitivity to adriamycin in adriamycin-resistant MCF-7 cells (Meslin et al., 2007b). Accordingly, breast tumors resistant to adjuvant chemotherapy express higher levels of PrP (Meslin et al., 2007a). Moreover, PrP is overexpressed and promotes cell survival in gastric cancer cells (Liang et al., 2006).
At the molecular level, PrP inhibits Bax-mediated cell death in human primary neurons, human differentiated neuronal NT2 teratocarcinoma cells, *Saccharomyces Cerevisiae*, and MCF-7 cells (Bounhar et al., 2006; Bounhar et al., 2001; Li and Harris, 2005; Roucou et al., 2004; Roucou et al., 2005). The anti-Bax function of PrP is physiological since PrP antisense constructs promote susceptibility to Bax-mediated cell death in human primary neurons (Bounhar et al., 2001). In human neurons and MCF-7 cells, PrP prevents the initial conformational change of Bax resulting in Bax translocation to the mitochondrial membrane, cytochrome c release and cell death. PrP is thus considered a *bona fide* Bax inhibitor (Roucou et al., 2005). The anti-apoptotic effect of PrP is specific to Bax since PrP does not inhibit Bak and Bid (Roucou et al., 2005). Despite being Bax-specific, PrP does not interact directly with Bax (Lin et al., 2008). This suggests that PrP requires an intermediate to carry out its anti-Bax function. This intermediate is most likely not a Bcl-2 protein because PrP can protect against Bax-mediated cell death in yeast, which are genetically deficient for Bcl-2 gene family members (Bounhar et al., 2006; Li and Harris, 2005). Yet the predominant anti-Bax form of PrP is the retrotranslocated cytosolic PrP (CyPrP) and not the more abundant cell surface GPI-anchored PrP (Lin et al., 2008).

Given the role of PrP in neuroprotection or survival of breast cancer cells, understanding the functional region or domain of PrP that is responsible for the anti-Bax function could provide a therapeutic target against neurodegenerative diseases and drug resistance in cancer cells. Structure-function analyses are often
useful in determining underlying molecular mechanisms of protein action through the identification of regulatory or functional domains. The structural domains susceptible to be involved in the anti-Bax function of PrP include the octapeptide repeat (OR) region (residues 51-91), and the globular C-terminal region of PrP. The ORs are implicated in copper binding (Brown et al., 1997a; Viles et al., 1999), as well as protection against oxidative stress (Dupiereux et al., 2007; Rachidi et al., 2003) and Doppel neurotoxicity (Drisaldi et al., 2004). Interestingly, embedded in this motif are four other octarepeats (residues 56-87) that show similarity to the Bcl-2 homology domain 2 (BH2 domain) of the anti-apoptotic Bcl-2 protein (LeBlanc, 1998). In Bcl-2, the BH2 domain is required for protection against Bax-mediated cell death (Yin et al., 1994) as are these BH2-like octapeptide repeats (BORs) since their deletion abolishes the anti-Bax function of PrP in human primary neurons (Bounhar et al., 2001). Moreover, both Bcl-2 and PrP are able to rescue PrP-null hippocampal cell lines against serum deprivation (Kuwahara et al., 1999) and PrP-null mice against N-truncated PrP-mediated toxicity (Nicolas et al., 2007). A second potential anti-Bax domain of PrP is its structured C-terminal region. This globular domain contains two β-strands and three α-helices (Donne et al., 1997; Riek et al., 1996; Zahn et al., 2000). Most of the familial human PrP mutations associated with prion diseases are localized in the globular domain. Some mutations can destabilize PrP structure (Liemann and Glockshuber, 1999; Swietnicki et al., 1998) or cause misfolding into a form partially resistant to PK digestion (Kiachopoulos et al., 2005; Vanik
and Surewicz, 2002), a property associated with the disease form of PrP (McKinley et al., 1983).

In this study, we investigated the role of the BORs and the globular domain of PrP in the anti-Bax function by generating a number of deletions constructs of PrP that eliminate various domains and tested the function of the mutant proteins against Bax-mediated cell death in the breast carcinoma MCF-7 cell line. While the BORs influence the ability of PrP to inhibit Bax-mediated cell death, the helix 3 is sufficient to confer anti-Bax activity in MCF-7 cells and in human neurons.

**Experimental procedures**

**Cell cultures**

Human primary neurons were cultured from fetal brains, obtained with ethical approval from the McGill University Institutional Review Board, as described previously (LeBlanc et al., 1997). All cell lines were obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells (Soule et al., 1973) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS, HyClone, Logan, UT). Mouse neuroblastoma N2a cells were cultured in Minimal Essential Medium (MEM) and 10% FBS.
Site-directed mutagenesis, cloning and sequencing of PrP mutants

All human PrP mutants carried a valine residue at position 129 and were cloned into the bigenic pBudCE4.1 vector (Invitrogen, Carlsbad, CA). EGFP or EGFP-Bax cDNA were subcloned under the EF-1α promoter of pBudCE4.1 and PrP cDNA was subcloned under the CMV promoter as described previously (Jodoin et al., 2007). The details of the primers, templates and techniques used to create the mutations are found in Table 1. Briefly, the mutants were either created by PCR amplification of a given region of PrP, which was then subcloned into pBud-EGFP or pBud-EGFP-Bax or generated by the Quikchange Site-Directed Mutagenesis protocol (Stratagene, LaJolla, CA).

Full-length PrP lacking all four BORs (PrPΔBOR4) has been described previously under the name PrPΔOR (Bounhar et al., 2001). The BH2 domain of the Bcl-2 protein was inserted in PrP in place of the BOR domain to create the PrPΔBOR4/BH2 mutant. CyPrPΔBOR1, CyPrPΔBOR2, CyPrPΔBOR3, and CyPrPΔBOR4 are CyPrP lacking one, two, three, or all four BORs. α-helices and β-strands from the C-terminal globular domain of PrP were sequentially removed from CyPrP to create the deletion mutants CyPrP23-227, CyPrP23-199, CyPrP23-172, CyPrP23-160, CyPrP23-143, and CyPrP23-127. K204, V210 or E219 were replaced by proline or alanine residues in CyPrP to generate K204P, V210P, E219P, K204A, V210A, and E219A. The PrP α-helix 3 with an added N-terminal Kozak sequence and methionine start codon was amplified by PCR and introduced in frame with the myc epitope and His tag of pBud-EGFP or pBud EGFP-Bax. A version
without tags was also produced. The PrP mutants were verified by sequencing at the Genome Quebec Innovation Centre sequencing platform (Montréal, QC).

**Molecular Modeling**

The 3D model of the globular domain of PrP showing the position of the K204, V210 and E219 amino acid residues was made using Pymol molecular visualization software (DeLano, 2002) (DeLano Scientific, Palo Alto, CA) and the Protein Databank file 1qlx (Zahn et al., 2000).

**Transfections**

MCF-7 cells were either plated on glass coverslips in 24-well plates (2.5x10^5 cells/well) for cell death assays or directly in 6-well plates (1.5x10^6 cells/well) for expression assays. Human primary neurons (1.5x10^5 cells) were plated on poly-L-lysine (Sigma-Aldrich, St-Louis, MI) coated plastic Aclar coverslips for cell death assays. N2a cells were plated directly in 6-well plates at a density of 0.8x10^6 cells/well for expression assays. The cells were transfected with 0.8μg of DNA for the 24-well plates or 4 μg of DNA for the 6-well plates with the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA). Alternatively, cells were transfected with the Helios Gene Gun system (BioRad, Mississauga, ON) as described previously (Jodoin et al., 2007). The DNA used for the transfections was purified with the UltraClean™ Endotoxin Removal Kit (MoBio, Carlsbad, CA) according to the manufacturer’s protocol.
Table 1. Mutagenesis and cloning methods used to generate the PrP mutants.

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</table>

**PrP mutants in pBud-EGFP:** 5'-CGACGGTCGACTCATAGAAGGCGGAAAGGCGCAGTGAGG-3' 5'-GCGCCAGACTCGAGGGTCGCTGATACG-3'
Cell death measurements

Twenty-four hours after transfection, cells were fixed in a solution of 4% paraformaldehyde (Sigma-Aldrich) and 4% sucrose (Bioshop, Burlington, ON) in phosphate-buffered saline (PBS: 150 mM NaCl, 2.7 mM KCl, 1.3 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$ pH 7.4) and the chromatin was stained for 20 minutes with 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich) in PBS. Cell death was identified as Hoechst-stained chromatin condensation in EGFP- or EGFP-Bax-positive cells by fluorescence microscopy (Nikon eclipse TE2000-U microscope, Mississauga, ON). The percentage of cell death was calculated as the number of EGFP-positive cells displaying condensed chromatin over the total number of EGFP-positive cells.

Subcellular fractionation

Subcellular fractionation of PrPΔBOR4 and wild-type PrP was performed as described previously (Jodoin et al., 2007).

Western blot analyses

Twenty-four hours after transfection, cells were either directly harvested or treated for 24h with 0.25 μM epoxomicin (BioMol, Plymouth Meeting, PA). Cellular proteins were extracted in non-ionic detergent Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP-40, 5 mM EDTA pH 8.0) for 10 min on ice. Detergent-insoluble proteins were separated by
centrifugation at 16,245 x g for 10 min at 4°C and insoluble proteins were resolubilized in 2% sodium dodecyl sulfate (SDS). Fifty or 100 µg of proteins, as quantified by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), were precipitated in four volumes of ice-cold 100% methanol overnight at -20°C. The proteins were centrifuged at 16,245 x g for 15 minutes at 4°C and resuspended in 20 µl Laemmli sample buffer (0.5% SDS (w/v), 1.25% β-mercaptoethanol, (v/v), 2.5% glycerol (v/v), 0.01% bromophenol blue (w/v), 15.6 mM Tris-HCl, pH 6.8). Proteins were submitted to 15% SDS-PAGE and transferred to PVDF membranes. The membranes were probed with the anti-PrP 3F4 (Kascsak et al., 1987) (1:1000), anti-β-actin (1:1000, Clone AC-15, Sigma, Oakville, ON), anti-mtHsp70 (1/1000, Clone JG1: Affinity Bioreagents, Golden, CO), and anti-GFP (1:1000, B-2, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Immunoreactivity was revealed with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and chemiluminescence reagents (Millipore, Billerica, MA).

**Statistical analysis**

Statistical analyses were done with StatView 5.0 software (SAS Institute Inc., Cary, NC) by an analysis of variance (ANOVA) followed by a Post-hoc Scheffé test. A p value under 0.05 was considered a significant difference.
Results

Substitution of the BOR domain of full-length PrP by the BH2 domain of the Bcl-2 protein does not rescue the anti-Bax function of PrPΔBOR4.

It was previously shown that the deletion of the BOR domain from full-length PrP (PrPΔBOR4) abolishes its anti-Bax function in human neurons (Bounhar et al., 2001). To investigate the role of the BOR domain in MCF-7 cells, the PrPΔBOR4 was subcloned under the CMV promoter in the bigenic vector pBudCE4.1 containing EGFP or EGFP-Bax cDNA under the eIF1-α promoter (Figure 4A). The expression of N-terminally EGFP-fused Bax is sufficient to activate Bax-mediated cell death and assures specific Bax activation in comparison to using an apoptotic insult that would activate several cell death pathways concomitantly. In transfected cells, PrPΔBOR4 migrates as several protein bands ranging from 23 to 36 kilodaltons (kDa) whereas full-length wild type PrP migrates mostly as a mature 36 kDa protein, similar to PrP from Syrian Hamster PrP transgenic mouse brain protein extracts (Figure 4B). This difference in glycosylation suggests that PrPΔBOR4 accumulates as an immature glycosylated protein. Neither PrP nor PrPΔBOR4 are toxic to MCF-7 cells (Figure 4C). Wild-type PrP significantly protects against Bax-mediated cell death in MCF-7 cells but PrPΔBOR4 does not (Figure 4C), consistent with our previous observations in human neurons (Bounhar et al., 2001). The loss of anti-Bax function in PrPΔBOR4 is not due to decreased retrotranslocation since cytosolic PrPΔBOR4 is easily detected in subcellular cytosolic and membrane fractions of
transfected cells (Figure 4D). Thus it is likely that the loss of function is attributable to the altered structure of the protein.

Next, we assessed if the loss of function in PrPΔBOR4 could be rescued by replacing the BORs by the BH2 domain of Bcl-2 (Figure 4A). The results show that PrPΔBOR4/BH2 does not rescue the loss of anti-Bax function in PrPΔBOR4 (Figure 4C) although western analyses suggest it is expressed at higher levels than PrP (Figure 4B) and is not cytotoxic (Figure 4C). This result shows that despite a similarity in their sequences, the BOR domain of PrP is not functionally similar to the BH2 domain of Bcl-2.

Partial but not complete deletion of BOR abolishes CyPrP anti-Bax function.

Because CyPrP is the form of PrP responsible for the anti-Bax function (Lin et al., 2008), we re-examined the involvement of the BOR domain in CyPrP’s anti-Bax function. We further tested if these repeats act in cooperation by sequentially deleting each repeat from CyPrP (Figure 5A). In transfected cells, each mutant migrates as a progressively shorter protein with more extensive deletions, as expected (Figure 5B). Deletion of one, two or three repeats eliminates the anti-Bax function (Figure 5A). In the case of CyPrPΔBOR1, cytotoxicity is also observed, thus it is difficult to determine the anti-Bax function for this mutant. Unexpectedly, the deletion of all four BORs retains the anti-Bax function (Figure 5A). This contrasts with the loss of anti-Bax function of PrPΔBOR4 in MCF-7 cells (Figure 4C) and in human neurons (Bounhar et al., 2001).
Figure 4. Expression and anti-Bax function of full-length PrPΔBOR4 and PrPΔBOR4/BH2 mutants.

A. Schematic diagram showing the main domains and structural elements of PrP, PrPΔBOR4 and PrPΔBOR4/BH2. Abbreviations: SP: N-terminal ER-targeting signal peptide (residues 1-23), BOR: BH2-like octapeptide repeats (residues 56-87), as opposed to standard octapeptide repeats (residues 51-91), STE: Stop-transfer effector sequence (residues 104-111), TM: transmembrane domain (residues 112-135), β1 and β2: beta-strands 1 (residues 128-131) and 2 (residues 161-164), α1, α2 and α3: alpha-helices 1 (residues 144-154), 2 (residues 173-194) and 3 (residues 200-227), GPI: glycosylphosphatidylinositol anchor signal (residues 232-253).

B. Western blot of 100 µg SDS-soluble proteins extracted from EGFP, PrP, PrPΔBOR4, PrPΔBOR4/BH2, or Mock-transfected cells with 3F4 anti-PrP and anti-β-actin antibodies. Proteins (1.05 µg) extracted from Syrian Hamster PrP transgenic mice brains (TgSHaPrP) were used as a positive control for PrP immunodetection.

C. Percentage of cell death assessed by chromatin condensation in MCF-7 cells transfected with pBud-EGFP (grey bars) or pBud-EGFP-Bax (black bars) vectors containing PrP, PrPΔBOR4 or PrPΔBOR4/BH2. Data represents the mean ± standard error of the mean (SEM) of 3 independent experiments. At least 100 cells were counted in each experiment. Asterisk indicates $p \leq 0.05$ compared to EGFP-Bax vector.

D. Western blot analysis of proteins from membrane and cytosolic subcellular fractions with 3F4, mtHsp70 and β-actin antibodies.
Figure 4.

A. 

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<td>PrPABOR4/BH2</td>
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<td></td>
<td>βH2</td>
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</table>

B. 

TgShaPrP  PrP  PrPABOR4  PrPABOR4/BH2  Mock

PrP

β-actin

C. 

- EGFP
- EGFP-Bax

% Cell death

Vector  PrP  PrPABOR4  PrPABOR4/BH2

D.

34 kDa  26 kDa

19 kDa  85 kDa  48 kDa

34 kDa  26 kDa

19 kDa  85 kDa  48 kDa

Membrane  Cytosol

PrP

mtHsp70

β-actin
Figure 5. Expression and anti-Bax function of CyPrP with deletion of one to four BORs.

A. Schematic diagram of the sequential CyPrP BOR deletion mutants showing the main domains and structural elements. Right panel: Percentage (mean and SEM) of condensed chromatin positive cells in transfected MCF-7 cells. Top line represents EGFP- or EGFP-Bax-transfected cells in absence of PrP constructs. Data represents results from six independent experiments. At least 200 cells were counted in each experiment. Asterisk indicates a statistically significant difference from pBud-EGFP or pBud-EGFP-Bax ($p \leq 0.05$).

B. Western blot of total proteins extracted from CyPrP and CyPrPΔBOR deletion mutants with 3F4 anti-PrP, anti-GFP and anti-β-actin antibodies.
Figure 5.

A.

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% Condensed chromatin

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<th>EGFP-Bax</th>
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B.

- PrP
- EGFP
- β-actin
Nevertheless, the loss of anti-Bax function in the partial BOR deletions indicates that this domain is involved in PrP’s anti-Bax function.

**Deletion of the C-terminal helix 3 abolishes CyPrP anti-Bax function.**

Mutants carrying sequential deletions of the cDNA regions encoding the 3 α-helices and 2 β-strands of PrP were tested (Figure 6A). None of the deletions causes toxicity in the cells (Figure 6A). Deletion of residues 200 to 231 from CyPrP (CyPrP_{23-199}) abolishes the anti-Bax function in MCF-7 cells (Figure 6A). Further deletions of α-helices 2 and 1 and β-strands 1 and 2 also eliminate PrP’s role against Bax-mediated cell death (Figure 6A). All the deletion mutants are highly expressed except for CyPrP_{23-127} (Figure 6C). Therefore, the loss of function is not due to faulty expression. These results indicate that residues 200 to 231 of CyPrP, which contain helix 3 and four non-helical C-terminal residues of CyPrP, are required for CyPrP’s anti-Bax function.

To verify if those four C-terminal residues are required for PrP’s anti-Bax function, we constructed a CyPrP_{23-227} mutant lacking these residues (Figure 6B). This deletion mutant is expressed in cells (Figure 6D) and retains the anti-Bax function (Figure 6B). Together, these results indicate that the helix 3 is required for the anti-Bax function of PrP.
Substitution of helix 3 K204, V210 and E219 amino acid residues by prolines causes a loss of CyPrP’s anti-Bax function.

To determine if the helical structure of helix 3 is necessary for the protection against EGFP-Bax-mediated cell death, amino acid residues at positions 204, 210 and 219 in CyPrP were substituted by a proline to disrupt its helical conformation (Figure 7A). These mutants represent a substitution in each turn of the helix, omitting the C214 residue involved in the disulfide bond and residues after E219 since the helical structure is already unstable beyond this point (Zahn et al., 2000). K204 and E219 are on the surface of the folded structure while V210 is part of the hydrophobic core of PrP (Figure 7B). Each of these mutants is expressed at equivalent levels in transfected cells considering their transfection efficiency determined by anti-GFP immunoblotting (Figure 7C). None of the mutants are toxic. The introduction of proline residues in K204, V210 and E219 results in a loss of anti-Bax function (Figure 7A). These results show that helix 3 stability is a requirement for CyPrP anti-Bax function.

Substitution of helix 3 residues by alanines shows that the N-terminal portion of helix 3 is required for CyPrP anti-Bax function.

Because disruption of the helix 3 region with prolines in CyPrP can affect the structure of the entire globular domain of CyPrP, we replaced helix 3 residues 204, 210 and 219 by alanines to further study the involvement of this helix 3 in the anti-Bax function. These mutations are predicted to have little effect on the structure of CyPrP (Blaber et al., 1993). However, replacing K204 by an alanine residue does break an ionic bond with E200, which destabilizes the structure of a
Figure 6. Deletions in the globular domain of CyPrP eliminate PrP’s anti-Bax function.

**A & B.** Schematic diagram of the sequential C-terminal CyPrP deletion mutants showing the main domains and structural elements. *Right panel:* Percentage (mean and SEM) of condensed chromatin positive cells in transfected MCF-7 cells. Data represents results from 4 to 5 independent experiments. At least 100 cells were counted for each experiment. Asterisk indicates a statistically significant difference from pBud-EGFP or pBud-EGFP-Bax (*p*≤0.05). **C.** Western blot analyses with 3F4 and β-actin antibodies of proteins extracted from cells transfected with CyPrP or CyPrP C-terminal globular domain deletion mutants. **D.** Western blot of total protein extracts from CyPrP23-231- or CyPrP23-227-transfected cells with 3F4, anti-GFP and anti-β-actin antibodies.
Figure 6.

A. % Condensed chromatin

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B. % Condensed chromatin

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C. [Image of Western Blot with bands for PrP and β-actin]  

D. [Image of Western Blot with bands for PrP and EGFP]
**Figure 7. Effect of CyPrP helix 3 missense mutations on the anti-Bax function of PrP.**

Schematic diagram of the CyPrP mutants with proline substitutions in helix 3. Right panel: Percentage (mean and SEM) of condensed chromatin positive cells in transfected MCF-7 cells. Data represents the results from 6 independent experiments. At least 100 cells were counted in each experiment. Asterisk indicates a statistically significant difference from pBud-EGFP or pBud-EGFP-Bax ($p \leq 0.05$). B. Location of the residues K204, V210 and E219 on the 3-dimensional structure of the CyPrP globular domain. C and D. Western blot analyses in protein extracts from cells transfected with CyPrP, the CyPrP proline substitution mutants (C) or the CyPrP alanine substitution mutants (D) with 3F4, anti-GFP or anti-β-actin antibodies. E. Percentage of cell death assessed by condensed chromatin in MCF-7 cells transfected with pBud-EGFP, pBud-EGFP/CyPrP, pBud-EGFP/CyPrP K204A, pBud-EGFP/CyPrP V210A, pBud-EGFP/CyPrP E219A, pBud-EGFP-Bax, pBud-EGFP-Bax/CyPrP, pBud-EGFP-Bax/CyPrP K204A, pBud-EGFP-Bax/CyPrP V210A, or pBud-EGFP-Bax/CyPrP E219A. Data represents the mean ± SEM of 3 independent experiments. At least 150 cells were counted in each experiment. Asterisk indicates a statistical difference from pBud-EGFP or pBud-EGFP-Bax-transfected cells ($p \leq 0.05$).
Figure 7.

A.

B.

C.

D.

E.
helix 3 peptide (Gallo et al., 2005). These alanine mutants are all expressed significantly (Figure 7D). The replacement of K204 by an alanine abolishes the anti-Bax function while the replacement of V210 and E219 by alanine residues has no effect (Figure 7E). These results show that the K204 amino acid residue of helix 3 is most important for the anti-Bax function.

Helix 3 of PrP encompasses the anti-Bax function in MCF-7 cells and in human primary neurons.

To assess if helix 3 is directly responsible for PrP’s anti-Bax function, we cloned residues 198 to 231 of PrP in frame with the myc and His tags (helix 3 tag) in pBud vectors and tested these constructs’ ability to prevent Bax-mediated cell death (Figure 8A). Similar to CyPrP, helix 3 tag significantly prevents Bax-mediated cell death in MCF-7 cells (Figure 8B).

The MCF-7 cells constitute a simpler system to perform structure-function analyses than primary human neurons. After we found the important anti-Bax region in helix 3, we verified if helix 3 could also prevent Bax-mediated cell death in primary human neurons (Figure 9). Similar to our observations in MCF-7 cells, the helix 3 tag prevents Bax-mediated cell death in human neurons (Figure 9B). To determine if helix 3 is functional in absence of the tags, we made a second construct without the myc and His tags (helix 3) (Figure 9A). Again, helix 3 inhibits Bax-mediated cell death. Together, these results show that helix 3 is sufficient for the anti-Bax function of PrP.
Discussion

In this manuscript, we conclude that the helix 3 is necessary and sufficient for the anti-Bax function of PrP. This conclusion is based on the fact that [1] deletion of the C-terminal 200-227 amino acids of PrP that encompass helix 3 results in a loss of anti-Bax function, [2] substitution of the K204, V210 and E219 amino acid residues with the helix disrupting proline amino acid residue destroys the anti-Bax function of PrP, [3] substitution of K204 with alanine also disrupts PrP anti-Bax function, and [4] expressing the helix 3 alone is sufficient to inhibit Bax-mediated cell death in MCF-7 cells and in primary human neurons. A search for similarities between the amino acid sequence of helix 3 and other proteins revealed only prion proteins from various species and no similarities between this sequence and other anti-Bax proteins. Therefore, the anti-Bax function of helix 3 is quite unique and differs from that found in a number of cytosolic anti-Bax proteins such as humanin, clusterin or Ku70 (reviewed by Walensky, 2006). However, involvement of helix 3 in PrP’s anti-Bax function is reminiscent of the survival or cell death role of α-helices in Bcl-2 family members (Walensky, 2006). Interestingly, deletion of amino acids 201-217 in mouse PrP containing a deletion of amino acid 23-88 results in a neurodegenerative disease including some nerve cell loss, cytoplasmic inclusions in enlarged neurons and excess endoplasmic reticulum characteristic of a neuronal storage disease (Muramoto et al., 1997). However, simply deleting the amino acids 23-88 does not induce neurodegeneration. Therefore, deletion of the anti-Bax function in helix 3 could be responsible for the observed pathologies in these mice, but only if PrP plays an
Figure 8. Effect of PrP helix 3 on the anti-Bax function of PrP in MCF-7 cells.

A. Schematic diagram of the helix 3 construct relative to the full length CyPrP. B. Percentage cell death assessed by condensed chromatin in MCF-7 cells transfected with pBud-EGFP (grey bars) or pBud-EGFP-Bax (black bars) alone, with CyPrP, or with the PrP helix 3 constructs. Data represents the mean ± SEM of 4 independent experiments. At least 100 cells were counted in each experiment. Asterisk indicates $p \leq 0.05$ compared to EGFP-Bax vector.
Figure 8.

A.

CyPrP

Helix 3 tag

B.

% Cell death

Vector CyPrP Helix 3 tag

EGFP EGFP-Bax
Figure 9. Effect of PrP helix 3 on the anti-Bax function of PrP in human primary neurons.

A. Schematic diagram of the helix 3 constructs with and without tags and the full length CyPrP. B. Percentage cell death assessed by condensed chromatin in human neurons transfected with pBud-EGFP (grey bars) or pBud-EGFP-Bax (black bars) alone, with CyPrP, or with the PrP helix 3 constructs. Data represents the mean ± SEM of 4 independent experiments. At least 50 to 100 cells were counted. Asterisk indicates $p \leq 0.05$ compared to EGFP-Bax vector.
Figure 9.

A. CyPrP Helix 3 tag Helix 3

B. % Cell death

Vector CyPrP Helix 3 tag Helix 3

EGFP EGFP-Bax
important physiological role in preventing Bax activation in neurons. Alternatively, the deletion mutant protein causes toxicity. Our findings warrant revisiting this model to elucidate the underlying molecular mechanism of this neurodegeneration.

The structural element that is necessary for helix 3 anti-Bax function is likely the helical structure itself. Disruption of the helical structure by the substitution of K204, V210 and E219 with the helix breaking proline amino acid eliminates the function of PrP against Bax-mediated cell death. With milder alanine substitutions, only the K204A mutant loses the anti-Bax function. This substitution disrupts the helical structure of the helix 3 peptide which shows structural autonomy (Gallo et al., 2005). In Gallo et al. (Gallo et al., 2005), it is believed that the K204 forms an ionic bond with amino acid residue E200, hence disruption of this bond could alter the structural features of the N-terminus of helix 3. However, the E200K familial PrP mutant with a Val at codon 129 (Val 129) retains the anti-Bax function of PrP entirely and the Met129 E200K PrP mutant retains partial function (Jodoin et al., 2007). This indicates that the disruption of the E200-K204 ionic bond may not be sufficient to explain the loss of helical structure and anti-Bax function. Alternatively, the K204 could be part of an amino acid motif that is responsible for PrP’s anti-Bax function. However, while K204 is entirely conserved in the PrP of all mammalian species and in chicken, amino acid variations are observed at V203, M205, M206 and E207 (Kim et al., 2008). Furthermore, the helix 3 V203I, R208H and E211Q familial PrP mutants retain the anti-Bax function of PrP (Jodoin et al., 2007), and the V210A and E219A PrP mutants studied in this paper also retain anti-Bax function.
in MCF-7 cells. Therefore, the results indicate that the K204 performs an essential function in PrP’s anti-Bax function. Exactly how the K204 amino acid residue functions will only be elucidated by identifying the molecular mechanism involved in PrP’s anti-Bax function. We have previously excluded a direct interaction of PrP with Bax and also other Bcl-2 family members (Bounhar et al., 2006; Lin et al., 2008). We can now use this sequence to search for interacting proteins that would mediate PrP’s anti-Bax function.

While the helix 3 is most important for the anti-Bax function of CyPrP, the N-terminal BOR domain also is implicated. Deletion of the four BORs does not affect the anti-Bax function of CyPrP, but deletion of the same BORs in the full length PrP loses the anti-Bax function in both human primary neurons and MCF-7 cells (Bounhar et al., 2001). We exclude the possibility that the loss of PrP anti-Bax function in PrP\(\Delta\)BOR4 is due to a defect of PrP retrotranslocation as previously shown with a number of Creutzfeldt-Jakob disease-associated PrP mutants (Jodoin et al., 2007). A number of factors may explain the difference between retrotranslocated and cytosolically expressed CyPrP\(\Delta\)BOR4. First, it is clear that the retrotranslocated, but not cytosolically expressed, CyPrP\(\Delta\)BOR4, is glycosylated. The glycosylation is expected since the epoxomicin proteasomal inhibitor added to increase the detection of CyPrP\(\Delta\)BOR4 blocks both the deglycosylation and degradation of retrotranslocated cytosolic proteins (Karaivanova and Spiro, 2000). Glycosylation may alter the structure of the protein resulting in a loss of the anti-Bax function of PrP. Second, different chaperones are involved in the folding of secreted proteins and cytosolically synthesized proteins (Ellgaard and Helenius, 2001). Therefore, it is possible that
PrPΔBOR4 is not folded in the same manner in those two subcellular locations. Nevertheless, deletion of only one, two or three of the four BORs in the CyPrP results in a loss of anti-Bax function indicating that this region influences the anti-Bax function. However, the C-terminal deletion constructs do not have any anti-Bax activity despite an intact BOR domain. One explanation for these results is that the N-terminal BOR region regulates the C-terminal helix 3 anti-Bax function. Indeed, it has been shown that the N-terminus of PrP regulates the C-terminal structure of PrP (Zahn et al., 2000). Together, the influence of complete or partial BOR deletions in either full length or CyPrP on the anti-Bax function of PrP is consistent with the role of the OR against Doppel- and ischemia-mediated cell death in vivo (Atarashi et al., 2003; Mitteregger et al., 2007).

The broad implications of these results are twofold. On one hand, elucidating the anti-Bax region of PrP provides a molecular handle upon which to develop therapeutic strategies against Bax in neurodegenerative diseases and an essential tool to further characterize the underlying molecular mechanism of PrP inhibition of Bax activation. On the other hand, helix 3 of PrP can be used to develop therapeutic strategies to prevent PrP’s anti-Bax function in cancer cells. Therefore, these results provide a two-prong approach against two equally devastating diseases.
CHAPTER 3: GENERAL DISCUSSION AND FUTURE DIRECTIONS
We aimed to find the role of the BOR and C-terminal domains of PrP in its protective function against Bax-mediated cell death. Our results show that both domains are involved, but in different manners. The BOR domain does not contribute to the anti-Bax function of PrP in the same way as the BH2 domain of Bcl-2 and needs to be undisrupted to participate in PrP anti-Bax function. However, we also found that CyPrP is able to protect cells against Bax with all four repeats removed, unlike what was observed for full-length PrP. On the other hand, the third and last alpha-helix of PrP is both necessary and sufficient to execute the anti-Bax function. Moreover, the N-terminus of helix 3 appears to be a possible active site for this function.

**Role of PrP helix 3 in anti-Bax function**

Helix 3 protects both human neurons and MCF-7 cells against Bax without support from the rest of the PrP protein. These findings corroborate earlier observations describing helix 3 structural autonomy (Gallo et al., 2005) and at the same time, these results rule out effects of the local interactions that maintain the three-dimensional structure of PrP. Thus, it is doubtful that the active site for PrP anti-Bax function is a combination of residues brought together by the folding of the globular domain. Of note, these results have implications for the protective function of the PrP homolog Shadoo. Most likely, the two proteins mediate their protective functions by different mechanisms since Shadoo has no region equivalent to helix 3 (Premzl et al., 2003; Watts et al., 2007). It could even be hypothesized that Shadoo has no anti-Bax activity. Hence, helix 3 must be able to
interact with the aforementioned intermediate required to inhibit Bax activation on its own.

Based on the point mutations done in this study, a probable site for this interaction would be the N-terminus of the helix, more specifically the lysine at position 204. Although it lost the anti-Bax function, the CyPrP K204A mutant should not have affected the helical structure as alanine residues have a high helical propensity. Furthermore, (Gallo et al., 2005), the loss of function of the K204A mutant is most likely not due to the disruption of the bridge formed with E200 (Figure 2D) since the familial mutant E200K also disrupts the salt bridge yet retains the anti-Bax function (Jodoin et al., 2007). In the complete PrP, K204 is facing towards the protein surface so that it is available for contact with the yet unidentified intermediate in the anti-Bax function. Nevertheless, further experimentation will be required to confirm the involvement of K204 in anti-Bax function.

Involvement of the BOR domain

The loss of function with only partial BOR deletion in CyPrP and with complete BOR deletion in full-length PrP indicates involvement of these repeats in protection. The functional independence of helix 3 and the dispensable nature of the complete BOR domain in CyPrP anti-Bax function imply that these repeats have a role secondary to that of helix 3. A plausible mechanism would have the BORs as an inhibitory domain to PrP’s anti-Bax function. Since the BORs are part of the flexible N-terminal tail, which can interact with the globular domain (Wells et al., 2006), it is possible that the repeats prevent the helix 3 region from
mediating PrP’s anti-Bax function and contacting the anti-Bax intermediate. In order to release helix 3 from this inhibition, its interaction with the BOR domain would have to be blocked by direct binding of the intact BOR domain by an interacting protein. In this model, the loss of function due to partial deletion would indicate that each BOR can independently inhibit the anti-Bax function and that the interactor protein cannot release helix 3 from inhibition because the BOR domain is incomplete. On the other hand, when all four BORs are deleted, the helix 3 region is free to mediate its anti-Bax function. The loss of function of PrPΔBOR4 in this model could be explained by a post-translational modification acquired in the ER that would prevent contact with the BOR interactor.

Such a modification could involve PNGase-mediated deglycosylation of PrP upon retrotranslocation. Figure 4D shows glycosylation on retrotranslocated PrPΔBOR4, this is due to the epoxomicin used to render the retrotranslocation of the protein detectable. As mentioned previously, epoxomicin inhibits PNGase-mediated deglycosylation (Karaivanova and Spiro, 2000). However, in the functional assays, no epoxomicin was used. Hence, retrotranslocated PrPΔBOR4, or any other retrotranslocated PrP, should be deglycosylated. In fact, the CyPrP mutant rescue experiments done in the laboratory (Jodoin et al., 2007; Lin et al., 2008) used unglycosylated PrP. The results of these studies support the idea that even though glycosylation tends to prevent proteasomal degradation, it is not required for protection against Bax. However, PNGase-mediated deglycosylation leaves an aspartate residue in place of the original asparagine residue so that N183 and N197 become D183 and D197 after going through the ERAD pathway. This
double mutation happens to all retrotranslocated PrP and in previous papers by our laboratory, this change did not seem to affect anti-Bax function (Jodoin et al., 2007; Lin et al., 2008). Yet it remains one of the few differences between retrotranslocated PrP and cytosol-endogenous PrP. It is possible that amino acid residues 183 and 197 play a role in the interaction between helix 3, the BOR domain and the BOR interactor protein, especially given the proximity of N/D197 to helix 3.

The putative inhibitory nature of the BOR domain seems to be contradicted by the numerous reports demonstrating the toxicity of the N-truncated PrP, which also lacks the repeats (Flechsig et al., 2003; Nicolas et al., 2007; Shmerling et al., 1998). Doppel lacks a BOR domain as well yet is also toxic to neurons (Li et al., 2000b; Moore et al., 1999; Moore et al., 2001; Rossi et al., 2001). That said, this observed toxicity may not be related to a loss of the anti-Bax function. However, deletion of Bax reduces sensitivity of Purkinje cells to Doppel in the Nagasaki PrP-null mouse (Heitz et al., 2007). Furthermore, the expression of Doppel fused to the N-terminus of PrP (residues 1-124) protects PrP-null hippocampal cell lines against serum deprivation (Lee et al., 2006). Yet in the same study, fusion of residues 1 to 95 of PrP, which contain the full BOR domain, did not give a protective effect to Doppel (Lee et al., 2006). In addition, truncation of the N-terminus of PrP has been shown to destabilize helix 3 (Zahn et al., 2000). As shown here, helix 3 stability is required for anti-Bax function (Figure 7). Hence, the adverse effects of N-truncated PrP and Doppel may be due to an effect on helix 3 stability and may not rule out the presence of inhibitory regulation within the N-terminus of PrP.
To precisely understand the role of these repeats, discovery of what differentiates full-length PrPΔBOR4-derived, ERAD-derived CyPrPΔBOR4 and direct CyPrPΔBOR4 is a useful approach. Another experiment to do would be the co-expression of the BOR domain with the helix 3 peptide to verify if the BOR domain has any influence on helix 3 anti-Bax function.

**Requirement for cytosolic PrP localization for anti-Bax function**

Most mutations in this study were performed in PrP lacking the ER-targeting and GPI anchor signal peptides, which is expected to give PrP exclusively in the cytosol. This system was chosen because CyPrP has been shown in our laboratory to be the main anti-Bax form of PrP through the correlation between loss of anti-Bax function and absence from the cytosol (Jodoin et al., 2007; Lin et al., 2008). In fact, PrP mutants lacking both signal peptides were able to rescue the anti-Bax function (Jodoin et al., 2007; Lin et al., 2008). However, no subcellular fractionation experiments had been done to prove that PrP without those signal sequences is actually localized to the cytosol. This argument does not question PrP retrotranslocation or direct translation of PrP with signal peptides in the cytosol. This argument does stress the importance of verifying the localization of the PrP 23-231 constructs commonly known as “CyPrP”. As a matter of fact, studies using a similar construct in N2a and PrP-null hippocampal cells found their version of PrP expressed in the nucleus (Crozet et al., 2006). Similarly, PrP lacking the N-terminal signal peptide was also found in that compartment (Nikles et al., 2008). Furthermore, two nuclear localization signals have been identified in PrP at positions 23-28 and 101-106 i.e. in positions
retained in the 23-231 constructs (Gu et al., 2003). Should the 23-231 constructs be found in the nucleus, the correlation between retrotranslocation and anti-Bax function would not be lost. Other retrotranslocated proteins have the nucleus as a final destination: EGF and the anti-Bax protein clusterin are both retrotranslocated and have both been observed in the nucleus (Jones and Jomary, 2002; Liao and Carpenter, 2007; Nizard et al., 2007; Zhang et al., 2005). In addition, PrP influences the expression of the genes encoding Bax and Bcl-2 (Chen et al., 2003; Du et al., 2005; Kim et al., 2004; Meslin et al., 2007b), hence a nuclear PrP may act as a transcription factor. Before further speculation, verification of the subcellular localization of PrP 23-231 is required. Should the construct be found in the nucleus, one must recall that all nuclear proteins are targeted to their final destination after synthesis in the cytosol (Kalderon et al., 1984). Furthermore, PrP 23-231 may still be able to mediate its anti-Bax function during its brief time in the cytosol, and despite its possible low cytosolic abundance should PrP 23-231 be more abundant in the nucleus.

Future directions

The goal of this study was to learn more about the mechanism by which PrP prevents Bax-mediated cell death through structure-function analyses. We identified a potential regulatory site in the BOR domain and a potential independent active site in the helix 3 region of PrP. Despite these findings, unanswered questions on the mechanism of PrP anti-Bax function linger. The nature of the intermediate between PrP and Bax remains unknown, but the helix 3 peptide, with or without mutations, can be used in cross-linking or yeast-two
hybrid experiments to identify candidate interacting proteins. The exact structure of the mutants used in this study can be deciphered with the usual NMR spectroscopy techniques but a combination of simpler techniques such as CD, the use of tryptophan fluorescence (Wildegger et al., 1999), and even molecular modeling could give early clues to changes in structure. Despite these unanswered questions, we have shown here that the helix 3 region of PrP may be the last in PrP structure, but it is far from being the least important in PrP anti-Bax function. The discovery of the implication of helix 3 in anti-Bax function is a step towards tighter and more specific regulation of Bax-mediated cell death in treatments against cancer and neurodegenerative diseases.
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APPENDICES
Appendix 1. Expression of helix 3 peptide in N2a cells

Expression of the tagged helix 3 peptide in cells was analyzed by western blot analysis with anti-His tag (1:200, Novagen, Madison, WI) and anti-β-actin antibodies in total proteins extracts. Proteins were obtained from cells transfected with 1, 2, 4 or 8 µg or either EGFP or PrP helix-tag cDNA.
Appendix 1.

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<th>µg DNA</th>
<th>EGFP</th>
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Histag | β-actin
Appendix 2. Research Compliance certification

January 15, 2008

Dr. Andréa LeBlanc  
Jewish General Hospital  
Dept. of Neurology & Neurosurgery  
3755 Cole Ste-Catherine  
Montreal, Quebec  
H3T 1E2

Dear Dr. LeBlanc:

We are writing in response to your request for continuing review for the study A01-M85-99 entitled "Function of prion protein against Bax-mediated cell death."

The progress report was reviewed and we are pleased to inform you that full Board re-approval for the study was provided on January 14, 2008, valid until January 13, 2009. The certification of annual review has been enclosed.

We ask you to take note of the investigator's responsibility to assure that the current protocol and consent document are deposited on an annual basis with the Research Ethics Board of each hospital where patient enrollment or data collection is conducted.

Should any modification or unanticipated development occur prior to the next review, please advise the IRB promptly.

Yours sincerely,

Roberta E. Menon, PhD  
Co-Chair  
Institutional Review Board

CC: A01-M85-99  
Ms. L. Martin – JGH
DATE OF I.R.B. APPROVAL
JAN 14, 2008

McGill Faculty of Medicine
Institutional Review Board
-Continuing Review-
Faculty of Medicine
McGill University

Principal Investigator: Andrea Iellanc
IRB Review Number: 061-M.R-99 Study Number (if any): Review Interval:

Title of Research Study: Function of prion protein in neuronal survival and cell death. Title changed to
Function of prion protein against Bax-mediated cell death (occurred when re-submitting but
grant is essentially the same).

Date of initial IRB approval: Jan 12, 2000 Date of previous continuing review (if applicable): Jan 12, 2006

INTERIM REPORT (PLEASE CHECK OR SPECIFY)

Current Status of Study:
Active Study: ACTIVE On Hold Closed to Enrollment:

Interim Analysis: Final Analysis: Study Not Activated:

If the study has not become active at McGill, please provide correspondence to explain: enclosed.

McGill hospital(s) where study has received approval of local Research Ethics Board(s) (if applicable):

JGH: X MCH: MGH: MNNHN:

RVH: __ SMH: Other:

McGill hospital(s) where study has not received approval of local Research Ethics Board(s) (if applicable): N/A

If study sponsorship or financial support has changed, please provide correspondence to explain: enclosed. Funded by
CIFHR #07-48594 starting 01/10/2001-2006 (did not renew but are still finishing a few manuscripts).

Number of subjects to be enrolled at McGill: N/A Number of McGill subjects enrolled to date:

N/A

Number of McGill subjects enrolled since last review: N/A Have McGill subjects withdrawn from the study?:

N/A

Has the study been revised since the last review?: No Have the study revisions been approved by the IRB?:

N/A

Has the consent form been revised since the last review?: No Date of the current consent form:

_  _

Are there new data since the last review that could influence a subject’s willingness to provide continuing consent?:

N/A

Have there been any serious adverse experiences (SAEs)? N/A

Have all serious adverse experiences (SAEs) and safety reports relevant to the study been reported to the IRB?:

N/A

SIGNATURES:

Principal Investigator: Andrea Iellanc Date: 19/02/2007

IRB Chair: Robert J. Salmon Date: Jan 14, 2008