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The prion protein interacts with Bcl-2 and Bax proteins

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Résumé

La fonction de la protéine normale du prion cellulaire (PrP^C), une protéine exprimée abondement dans le cerveau, est présentement inconnue. Les quatre répétitions octapeptides de PrP^C ont une séquence homologue au domaine fonctionel BH2 (Bcl-2 Homology 2) des protéines Bcl-2 et Bax. Bcl-2 et Bax sont responsables pour la régulation de l'apoptose. Le domaine BH2 de Bcl-2 est responsable pour la fonction anti-apoptotique de la protéine Bcl-2 ainsi que l'intéraction protéique entre Bcl-2 et Bax. L'intéraction protéique entre Bcl-2 et Bax, une protéine qui induit la mort cellulaire, assure la survie cellulaire. Dès que cette intéraction est interrompue par des mutations dans le domaine fonctionel BH2 de Bcl-2 ou s'il y a un déséquilibre protéique, les cellules meurent. Puisque le domaine BH2 est présent en cinq copies chez le prion, on propose que PrP^C peut être impliqué dans la survie ou mort neuronale par un mécanisme semblable au Bcl-2 et la famille de protéines reliées au Bcl-2. Cette thèse étudie les intéractions entre les protéines humaines PrP^C, Bcl-2, et Bax dans les neurones humaines primaires ainsi que des extraits de cerveau humain foetal et adulte par des méthodes de "crosslinking" et "co-immunoprecipitation". L'intéraction protéique entre PrP^C et Bcl-2 a été aussi étudié par le "yeast-two-hybrid matchmaker". Nos résultats indiquent une intéraction entre PrP^C et Bcl-2 ainsi que PrP^C et Bax *in vivo* et *in vitro*. On a aussi étudié l'expression de PrP^C, Bcl-2, et Bax dans le cervelet humain foetal ainsi qu'adulte. Dans le système nerveux central qui vieillit, PrP^C peut acquérir les fonctions de Bcl-2 ou Bax. Nos résultats indiquent que l'expression de PrP^C augmente avec l'age. Au contraire, l'expression de Bcl-2 et Bax diminuent avec l'age. D'après les résultats obtenus, on démontre que PrP^C interagit avec Bcl-2 et Bax. Des mutations, la sur-expression, ou l'isoforme conformationelle anormale du prion, peuvent avoir un effet sur ces intéractions. Si les intéractions protéiques entre PrP^C avec Bcl-2 ou Bax, sont nécéssaires pour la survie des neurones, une interruption de celles-ci peut expliquer les processus

moléculaires sous-adjacent à la neurodégénération associée à la maladie du prion.

Abstract

The function of the normal cellular human prion protein (PrP^C), a highly expressed protein of the brain, is presently unclear. There is amino acid sequence homology between four octapeptide repeats localized in the NH_2 -terminus of PrP^C, and the BH2 functional and interacting domain of Bcl-2 and Bax, key modulators of apoptosis. The BH2 domain confers Bcl-2's anti-apoptotic function and allows its heterodimerization with the cell death protein Bax. In a cell, when Bcl-2 interacts with itself as a homodimer, no apoptosis occurs. In contrast to Bcl-2, overexpression of Bax as a monomer or homodimer results in cell death. Protein-protein interactions between Bcl-2 and Bax rescue cells from death. The ratio between Bax-Bax homodimers versus Bcl-2-Bax heterodimers determines the viability of cells following an apoptotic stimulus. As soon as the Bcl-2-Bax interactions are disrupted either by mutations, or an imbalance in Bcl-2/Bax ratio, cells undergo apoptosis.

Based on the amino acid sequence homology between PrP^C, Bcl-2, and Bax proteins, we propose that PrP^C may be implicated in modulating neuronal survival and death as a member of the Bcl-2 family of proteins. The present study investigates interactions between human PrP^C, Bcl-2, and Bax in human primary foetal neurons as well as in human foetal and adult brains by crosslinking and co-immunoprecipitation. The PrP^C-Bcl-2 interaction was also tested by the yeast-two-hybrid system. Results indicate an interaction between PrP^C and Bcl-2 as well as PrP^C and Bax *in vivo* and *in vitro*. In addition, the expression levels of PrP^C, Bcl-2, and Bax were studied in the cerebellum of human foetal and adult brain. Results show that the levels of Bcl-2 and Bax decrease with age whereas the levels of PrP^C increase. In the aging central nervous system, PrP^C may functionally replace Bcl-2 or Bax. Overexpression or different modifications of PrP^C such as conformational change and mutations may disrupt the protein-protein interactions between PrP^C, Bcl-2, and Bax. If PrP^C-Bcl-2/Bax interactions function in neuronal

survival, disruption of these interactions could explain the underlying molecular mechanism of neuronal death in prion diseases.

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Abbreviations

AP buffer	Alkaline phosphatase buffer
Bcl-2	B-cell lymphoma-2
BCA	Bicinchinonic acid
BCIP	5-Bromo-4-chloro-3-indoyl-phosphate
BH2	Bcl-2 homology 2 domain
BSE	Bovine spongiform encephalopathy
Ca^{2+}	Calcium
CJD	Creutzfeldt-Jakob Disease
CNS	Central nervous system
COOH	Carboxyl-terminus
DSP	Dithiobis-succinimidyl-proprionate
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FASE	Familial Atypical Spongiform Encephalopathy
FFI	Fatal Familial Insomnia
GPI	Glycosyl phosphatidyl inositol
GSS	Gerstmann-Straussler Syndrome
IP	Immunoprecipitation
kDa	Kilodalton
µl	Microliters
µg	Micrograms
ml	Milliliters
mRNA	Messenger ribonucleic acid
NBT	Nitro-bromo tetrazolium chloride
NGF	Nerve growth factor
NH_2	Amino terminus
ONPG	Orthonitrophenyl- galactose

OPTI-MEM	Optimal Minimal Essential Medium
PCR	Polymerase chain reaction
PIPLC	Phosphatidyl inositol phospholipase C
PrP^{C}	Normal cellular prion protein
PrP^{SC}	Scrapie prion protein
ROS	Reactive oxygen species
SD	Synthetic dropout media
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
STE	Stop transfer effector sequences
TM	Transmembrane domain
Tris-HCl	Trizma base hydrochloride
TUNEL	Terminal transferase-mediated dUTP nick end labeling

I Introduction

In 1982 Stanley B. Prusiner defined "prions" as proteinaceous infectious particles whose overall properties differ from conventional pathogens such as bacteria, fungi, parasites, and viruses. Since then, there has been puzzle and controversy in the scientific field regarding the fact that a protein with unusual biological and structural properties is able to cause transmissible diseases without the involvement of nucleic acid (reviewed by Prusiner, 1982; Prusiner, 1991; Prusiner, 1996). At present times, prion diseases ranging from Mad Cow Disease to "new variant" Creutzfeldt-Jakob Disease are of great interest in the neuroscience field. It is hypothesized that the fundamental event underlying the pathogenesis of prion diseases of both animals and humans is the conformational modification in a normal cellular protein (PrP^{C}) into its pathogenic isoform (PrP^{SC}) (reviewed by Prusiner, 1982; Prusiner, 1990; Prusiner and Westaway, 1991; Prusiner, 1996; reviewed by Aguzzi and Weissman, 1997) . Despite considerable advances in the prion field and the elucidation of the prion protein's molecular genetics, the function of the normal cellular prion protein (PrP^{C}) is unclear. Several reports suggest a role for PrP^{C} in lymphocyte proliferation, calcium regulation, synaptic transmission, neuronal differentiation, and central nervous system (CNS) maturation (Cashman et al., 1990; Manson et al., 1992; Forloni et al., 1993, 1994; Collinge et al., 1994). Interestingly, four octapeptide repeats in the NH_2-terminal region of the prion protein are highly homologous to the BH2 anti-apoptotic functional and interacting domain of Bcl-2 and Bax, two key modulators of apoptosis. The homology existing between PrP^{C}, Bcl-2, and Bax proteins is significant and indicates a possible membership of PrP^{C} to the family of Bcl-2 proteins. Bcl-2 and related proteins are implicated in the regulation of apoptosis. Their mechanism of action is dependant on protein-protein interactions amongst themselves. As a member of the Bcl-2 family, PrP^{C} may modulate neuronal death and survival in a similar mechanism. The present study investigates

the protein-protein interactions between PrP^C, Bcl-2, and Bax in primary human foetal neurons, foetal and adult human brain for the first time.

1. Prion Diseases of humans and animals

Prion diseases or transmissible spongiform encephalopathies represent a group of unusual and rare fatal neurodegenerative disorders affecting both human and animal species (reviewed by Prusiner, 1982; Prusiner, 1990; Prusiner and Westaway, 1991; Prusiner 1996). The animal forms of prion diseases are all transmissible and include scrapie in sheep and goats, bovine spongiform encephalopathy, feline spongiform encephalopathy, mink encephalopathy, as well as chronic wasting of deer and elk. Bovine spongiform encephalopathy (BSE), also known as Mad Cow Disease is the most worrisome of animal prion disorders (Collinge et al., 1996; reviewed by Collinge, 1997). Recently, interest in Mad Cow Disease has reached a climax because of the epidemic in the United Kingdom and now in European countries. The possibility that BSE may pose a significant threat to public health through dietary exposure to BSE-infected meat has attracted a great deal of attention to prion diseases in general (reviewed by Collinge, 1997).

Scrapie was the first prion disease identified among animals over two hundred years ago (McGowan, 1922; Prusiner, 1991; reviewed by Prusiner, 1996). The disease was acquired through consumption of contaminated feed. Afflicted sheep developed loss of coordination, dementia, and an intense itch causing them to scrape off their wool as a result (McGowan, 1922; Cuille et al., 1936; Prusiner 1991; reviewed by Prusiner, 1996). In addition, the brains of the affected animals showed spongiform encephalopathy (McGowan, 1922; Cuille et al., 1936; reviewed by Prusiner, 1996). Transmissibility of scrapie by intracerebral inoculation to hamsters was demonstrated in 1936. Interestingly, the transmissible infectious agent, which could not be isolated at the time, was resistant to all agents known to disrupt nucleic acids (ie. ultraviolet or ionizing radiation, nucleases,

detergents) (Cuille et al., 1936; Alper et al., 1967; Prusiner et al., 1982; Bolton et al., 1982; McKinley et al., 1983; Prusiner et al., 1984; reviewed by Prusiner et al., 1984; reviewed by Prusiner, 1996).

Several years following the scrapie outbreak, the existence of transmissible human prion diseases became evident. Kuru, a clinically and pathologically similar disease to scrapie, was the first transmissible human prion disease discovered (Gadjusek and Zigas, 1959; Gadjusek et al., 1966; Prusiner, 1991; reviewed by Prusiner, 1996; reviewed by Aguzzi and Weissman, 1997; reviewed by Collinge, 1997). In the 1950's, Vincent Zigas and Carleton Gadjusek studied the Fore tribe in New Guinea whose members were dying of a disease characterized by progressive cerebellar ataxia and dementia (Gadjusek and Zigas, 1959; Gadjusek et al., 1966; Prusiner, 1991; reviewed by Prusiner, 1996). The Fore tribe members developed kuru as a result of consumption of infected offals from deceased individuals . This event was part of a cannibalistic ritual to honour their ancestors. Examination of the brains of afflicted individuals revealed spongiform encephalopathy, a feature reminiscent of scrapie (Hadlow, 1959; Gadjusek et al., 1966; Prusiner, 1991; reviewed by Prusiner, 1996). In 1966, transmission of kuru to chimpanzees in 1966 by intracerebral inoculation, revealed that the infectious agent, as that of scrapie, was devoid of nucleic acid. It was then evident that both scrapie and kuru shared the same causative agent (Gadjusek et al., 1966; Prusiner, 1982; 1991; reviewed by Prusiner, 1996). In 1982, isolation of the infectious agent enriched in scrapie-infected hamster brains, revealed that a protein was responsible for the prion diseases. On the basis of amino acid sequence analysis, the causative protein was defined as an infectious abnormal isoform of a normal cellular glycoprotein (PrP^{C}). The abnormal protein was designated scrapie prion (PrP^{SC}) (Bolton,et al., 1982; Prusiner et al., 1982; Prusiner and Westaway, 1991; reviewed by Prusiner 1996).

1.1 Forms of human prion disorders: acquired, sporadic, inherited

Although kuru is now almost extinct from society due to cessation of cannibalism, other human prion disorders such as Creutzfeldt-Jakob (CJD), Gerstmann-Straussler Scheinker Syndrome (GSS), Fatal Familial Insomnia (FFI), and variant forms of these continue to represent concern. In addition to being transmissible, human prion diseases occur in acquired, sporadic, or inherited forms, affecting one person per million per year (Prusiner and Westaway, 1991; Prusiner, 1994; reviewed by Prusiner, 1996; reviewed by Aguzzi and Weissman, 1997; reviewed by Collinge, 1997).

1.1.1 Acquired and sporadic human prion disorders

Human prion diseases that emerge sporadically or are acquired iatrogenically include kuru and 85% of CJD cases (reviewed by Prusiner, 1996). Infectious prion diseases arise by a species-specific interaction between exogenous PrP^{SC} and endogenous PrP^{C}. Iatrogenic transmission of CJD is mainly through use of contaminated surgical instruments, corneal transplantation, or intramuscular injection of cadaveric human pituitary-derived growth hormone (gonadotrophin) (Weller et al., 1989; Collinge et al., 1991; Collinge et al., 1996; reviewed by Collinge, 1997). Iatrogenic CJD cases are quite rare. The majority of CJD cases are sporadic and do not involve any mutations in PrP^{C} or iatrogenic exposure (Palmer et al., 1991; Baker et al., 1992; Parchi et al., 1996). The etiology of such cases is unclear.

1.1.2 Inherited human prion disorders

Inherited human prion diseases include the remaining 15% CJD cases, all GSS cases, all FFI cases, as well as variants of these that are continuously being identified. Inherited human prion diseases are associated with coding mutations in the prion protein gene which are inherited in an autosomal dominant pattern (reviewed by Prusiner, 1994). Inherited cases are presumed to involve a spontaneous transformation of mutant PrP into PrP^{SC}. There are nineteen different

mutations associated with prion disease phenotypes identified so far which include 1) point mutations within the coding sequence resulting in single amino acid substitution in PrP^C, 2) missense mutations, and 3) insertions encoding copies of octapeptide repeats located in the N-terminus of PrP^C. The discovery that mutations were involved in prion disease allowed the establishment of genetic linkage to disease and allowed the identification of individuals at risk (Collinge et al., 1989; 1991; Tateishi et al., 1995; reviewed by Collinge, 1997).

1.1.3 Genetic predisposition of prion diseases

Genetic susceptibility and age of onset relevant to acquired, sporadic, and inherited prion diseases are determined by a common polymorphism of the human prion protein present at residue 129 (Baker et al., 1991; Owen et al., 1990; reviewed by Collinge, 1997). In the general population, either a methionine or valine is found at position 129. Approximately 51% Caucasians are heterozygotes, 38% are homozygous for the more frequent methionine allele, and 11% are homozygous for valine. Homozygosity for either amino acid predominates in sporadic CJD cases whereas homozygosity for valine is more prevalent in iatrogenic CJD cases (Baker et al., 1991; Palmer et al., 1991; Collinge et al., 1991; reviewed by Collinge, 1997). A protective effect of heterozygosity is seen in inherited forms of prion diseases where the age of onset is 1-2 decades later in heterozygotes than in homozygotes (Baker et al., 1991; reviewed by Collinge, 1997). Normally, prion diseases manifest in the fourth decade of life with the exception of sporadic CJD which peaks at 60 years of age. However, there have been recent reports of younger individuals affected. In particular, the new variant of CJD identified among twelve teenagers in Great Britain has recieved much attention due to the possibility that the individuals acquired the prion disease as a result of eating contaminated beef products . Individuals affected by new variant CJD are also homozygous for methionine at codon 129 (Collinge et al., 1996; reviewed by Collinge, 1997).

1.2 Pathological and clinical hallmarks

Pathological hallmarks of both animal and human prion diseases include variable degrees of spongiform encephalopathy, neuronal loss, plaque and amyloid fibril formation, and astrocyte gliosis (Beck and Daniel, 1987; reviewed by Prusiner, 1996; reviewed by Aguzzi and Weissman, 1997; reviewed by Collinge, 1997). Clinically, prion diseases are characterized by ataxia, and motor and cognitive abnormalities that progressively lead to dementia and provoke death within a few weeks to ten years prior to infection (Parchi et al., 1996; reviewed by Prusiner, 1996). However, the clinical manifestations, the disease duration, age of onset, as well as the type and distribution of neuropathological lesions vary with the different phenotypes associated with human prion disorders CJD, GSS, FFI, and FASE (Familial Atypical Spongiform Encephalopathy). The more common conditions of sporadic and familial CJD are characterized by extrapyramidal, pyramidal, and cerebellar ataxia, and myoclonus with a peak onset at 60 years of age while GSS is described as ataxia with progressive dementia occurring after the age of 40. In addition, GSS is associated with amyloid plaques or neurofibrillary tangles in brains of affected individuals (Collinge et al., 1989; Hsiao et al., 1990; Prusiner, 1994; Ghetti et al., 1996). In contrast to both CJD and GSS, FFI is characterized as a thalamic dementia associated with insomnia (Lugaresi et al., 1986; Medori et al., 1992; Goldfarb et al., 1995). Recently, our laboratory in collaboration with a Brazilian research group headed by Ricardo Nitrini identified a familial atypical spongiform encephalopathy (FASE) associated with atypical neuropathological features. FASE is characterized by severe neuronal loss in the cerebellum and in the putamen and minimal gliosis in the affected areas (Nitrini et al., 1997). The different phenotypes that arise in prion diseases are thought to result from the existence of diverse prion strains, an important aspect covered later in this discussion.

1.2.1 Molecular mechanisms of prion diseases: models for prion transmission

The exact mechanism by which prion infectivity increases is unknown. However, several models have been proposed including: 1) the "folding" model ("protein only" hypothesis) proposed by Stanley Prusiner, 2) the "seeding" model, 3) the "unified" theory, and 4) the "unconventional virion" model (Prusiner, 1991; reviewed by Prusiner, 1996). The most accepted molecular model explaining the process of prion transmissibility is the "folding" model proposed by Stanley B. Prusiner. The "folding" model also known as the "protein only" hypothesis is depicted in figure 1. According to the "protein only hypothesis", conformationally abnormal PrP (PrP^{SC}) arising spontaneously or through mutations, binds to normal cellular PrP (PrP^{C}) to induce a conformational change such that PrP^{C} flips from its normal α-helical conformation and acquires the misfolded β-sheet conformation of PrP^{SC} (Griffith, 1967; Cohen et al., 1994; Prusiner 1987a, 1987b, 1991, 1993; Prusiner and DeArmond, 1990; Prusiner and Westaway, 1991; reviewed by Prusiner, 1996; reviewed by Horwich and Weissmann, 1997). The multiplication of prion infectivity is an exponential process where PrP^{SC} combines with one PrP^{C} molecule to produce a heterodimer which transforms into two PrP^{SC} molecules (Prusiner, 1991; reviewed by Prusiner, 1996; reviewed by Horwich and Weissmann, 1997). The newly formed PrP^{SC} molecules combine with two PrP^{C} molecules and give rise to four PrP^{SC} molecules and so on. Repeated cycles of the continuous conversion of PrP^{C} to PrP^{SC} may account for PrP^{SC} propagation and clinical progression of prion diseases (within weeks to months after infection) (Prusiner, 1991; reviewed by Prusiner, 1996; reviewed by Horwich and Weissmann, 1997). Prion diseases are characterized by the accumulation of the abnormal PrP^{SC} in the CNS which results in the enlargement of the endoplasmic reticulum, vacuolization of neurons, and spongiform encephalopathy. The conversion of PrP^{C} to PrP^{SC} occurs inside neurons and PrP^{SC} accumulates in the lysosomes. Filled lysosomes eventually breakdown, hydrolytic enzymes are

released, and cells are damaged as a result. As diseased cells die, infectious prion particles are released and attack more cells (Borchelt et al., 1992; Laszlo et al., 1992). This hypothesis is not yet proven.

Recently, Stanley Prusiner and colleagues reported that a yet uncharacterized protein (protein X) serves as an intermediate in the conversion of PrP^{C} to PrP^{SC} (Prusiner et al., 1990; 1992; Telling et al., 1995; Kaneko et al., 1997). Kaneko et al (1997) identified amino acids 167- 218 in the carboxyl terminus of PrP^{C} as the binding site for protein X. Deletion of amino acids 167-218 prevents PrP^{SC} formation (Prusiner et al., 1990; Telling et al., 1995; Kaneko et al.,1997). In addition, foldases or chaperones (heat shock proteins) may also facilitate the PrP^{C} to PrP^{SC} conversion (Prusiner et al., 1990; Edenhofer et al., 1996). Interactions between PrP^{C} and Hsp60, one of the best characterized members of molecular chaperones involved in ATP-mediated protein folding support this notion (Edenhofer et al., 1996).

A second model explaining the transmissibility of prion diseases is the "seeding" model (see figure 2). The "seeding model" implies that the PrP^{C} to PrP^{SC} conformational conversion is a thermodynamically controlled and reversible process (Come et al., 1993). PrP^{SC} acts as a seed for its own propagation. PrP^{SC} aggregates form upon the PrP^{C} molecule in a crystal-like array (Griffith et al., 1967; Come et al., 1993). As more aggregates of PrP^{SC} accumulate and deposit in cells, the PrP^{C} to PrP^{SC} conversion occurs (Griffith et al., 1967; Caspar et al., 1980; Come et al., 1993; reviewed by Horwich and Weissmann).

The "unified" model proposed by Charles Weissmann postulates that the infectious agent isolated from the brains of diseased animals consists of both a protein (PrP^{SC} or apo-prion) and a viral nucleic acid (co-prion) (Weissmann et al., 1991) . The "unified" theory incorporates some of the basic features of the "protein only" hypothesis (Weissmann et al., 1991). For instance, the protein

MODEL EXPLAINING THE TRANSMISSIBILITY OF PRION DISEASES

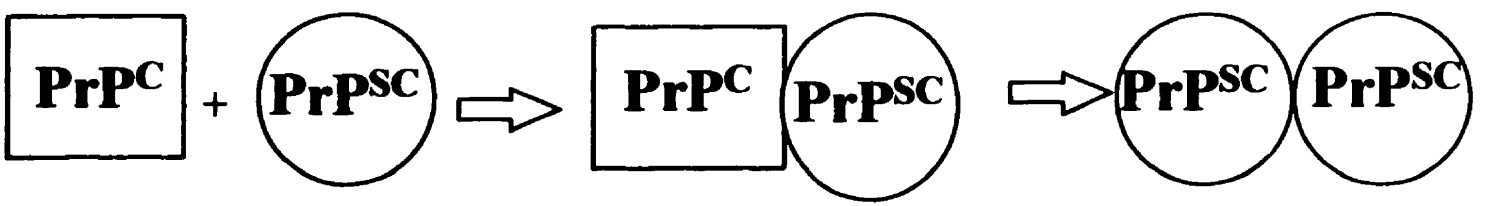


Figure 1. - "Folding model" explaining the transmissibility of prion diseases

(Prusiner, (1991), Science 252, p.1515-22)

THE SEEDING MODEL

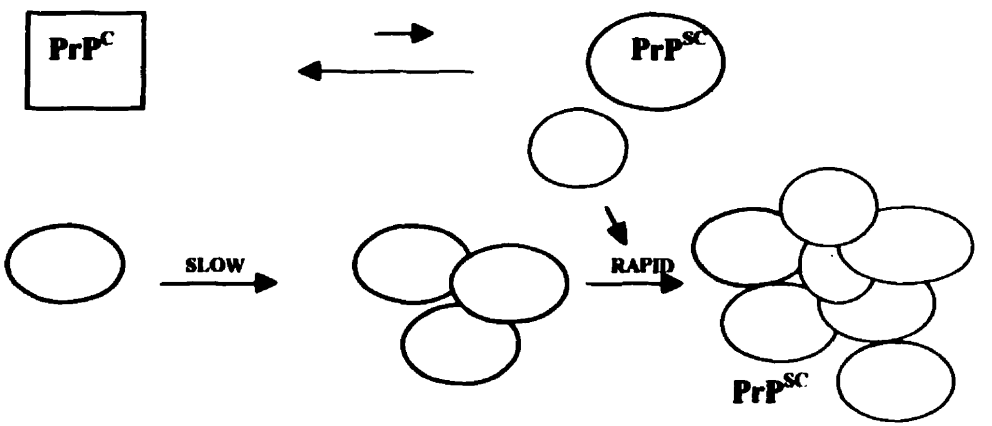


Figure 2. - The seeding model explaining prion propagation

Reference: Come et al., 1993.

component of the holoprion, PrP^{SC} (apo-prion), can cause disease even in the absence of nucleic acid (Weissmann et al., 1991). The unified theory also associates PrP^{SC} with nucleic acid. PrP^{SC} enters the cell and possibly recruits a nucleic acid which serves as co-prion. Co-prion is replicated by the cellular enzymes and multiplies at a considerably high rate. By this manner, the PrP^{SC} molecule propagates itself causing disease as a result (Weissmann, 1991).

Finally, reknown virologists argue that a hidden virion particle (nucleic acid coated by protein) may be responsible for establishing infection in hosts (Manuelidis et al., 1995). The virion theory is not conclusive since the infectious agent causing prion diseases remains infectious even after treatment with agents that disrupt nucleic acid and that would normally eliminate viral infectivity (Alper et al., 1967; Prusiner et al., 1982; McKinley et al., 1983).

1.2.2. The concept of "species barrier"

Transmission of prion diseases between different mammalian species is limited by a species barrier. The existence of the species barrier accounts for the increased incubation periods between initial infection and clinical manifestations of prion disease as well as even complete resistance to transmission of disease between some species. A large body of evidence accumulated from transgenetic and in vitro PrP^{C} to PrP^{SC} conversion studies, indicate that the key determinants of the species barrier are the degree of homology between the PrP molecules of the host and that of the inoculum (Carlson et al., 1994; reviewed by Prusiner, 1996; Lasmezas et al., 1997) . The PrP^{C} to PrP^{SC} conversion occurs most efficiently when the PrP molecules of the host and that of the inoculum are homologous (Carlson et al., 1994; reviewed by Prusiner, 1996; Lasmezas et al., 1997). At present time, an issue of great concern in the medical field is the remote possibility of overcoming the species barrier especially the one existing between cattle and humans (Collinge,1996).

2. The human prion protein gene (PrP)

2.1 Structure of the PrP gene

There is a conservation of the open reading frame of the PrP gene among mammals ranging from mouse, hamster, chicken, sheep, to human species (Liao et al., 1986). Homologous PrP sequences have also been detected in Drosophila and lower eukaryotes (Liao et al., 1986; Westaway, 1986). Localization of the human PrP gene is to the short arm of chromosome 20 in humans (Oesch et al., 1985; Robakis et al., 1986; Puckett et al., 1991; Liao et al., 1986). The structure of the human PrP gene is depicted in figure 3. The human PrP gene spans 16 Kb and is contained between two exons separated by a 10 Kb intron. Exon 1 (approximately 136 bp long) encodes a portion of the 5' untranslated leader sequence (Kretzschmar et al., 1986; Puckett et al., 1991). The complete open reading frame of 759 nucleotides is contained within the second exon, eliminating the possibility that variant forms of PrP can arise via alternative splicing. The first and second exons of the PrP gene are transcribed into a 2.1 Kb single mRNA species and exon 2 encodes the open reading frame of a 253 amino acid normal cellular sialoglycoprotein designated PrP^C (Robakis et al., 1986; Kretzschmar et al., 1986).

2.2. PrP mRNA and protein expression

Northern blot analysis indicate that the 2.1 Kb PrP mRNA species is highly expressed in the pyramidal and dentate granule cells of the hippocampus, Purkinje cells of the cerebellum, and in the large neurons of the cortex, medulla, and septum in adult brain (Manson et al., 1992). PrP mRNA expression is regulated during the development of the central nervous system of rodents (Moser et al., 1994; Manson et al., 1992). Low levels of PrP mRNA have been detected at birth but gradually increase in hamster and mouse brain during the immediate postnatal period to a level maintained throughout adult life (Mobley et al., 1988; Manson et al., 1992). The pattern of PrP gene expression in the human central nervous system is

PRION PROTEIN GENE STRUCTURE

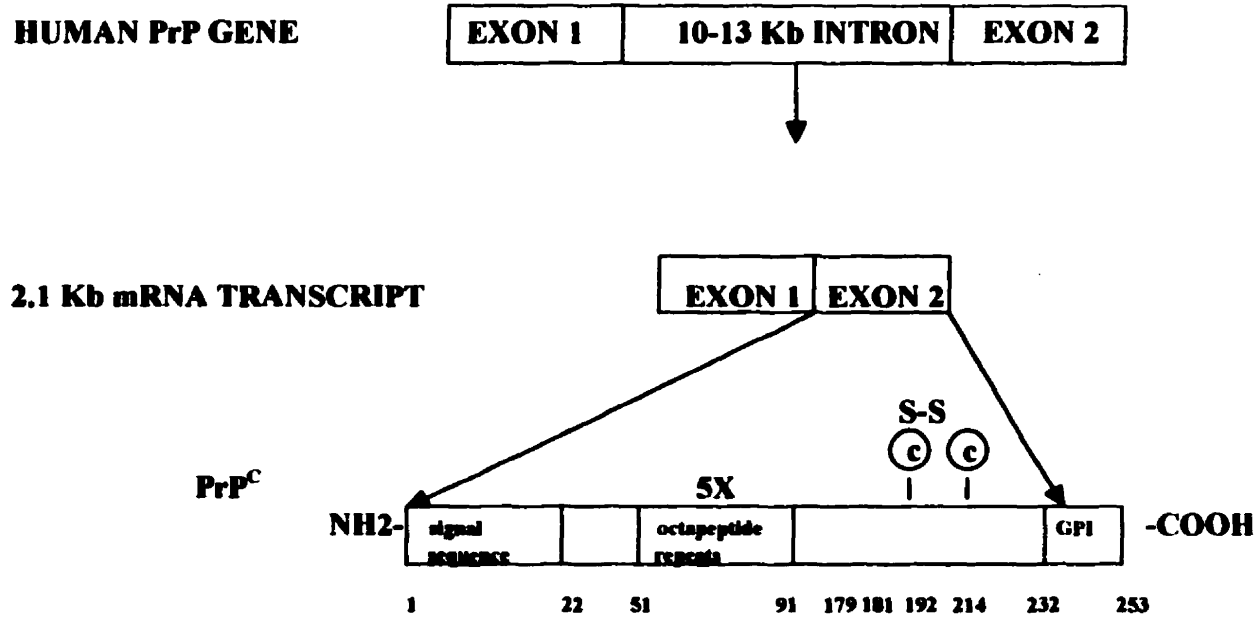


Figure 3. - Structure of the human PrP gene

Reference: Puckett et al., 1994.

unknown. The DNA sequences and factors involved in the regulation of PrP gene expression in CNS neurons have not been identified. A study done in murine neuronal cells implies that nerve growth factor (NGF) may upregulate PrP mRNA expression but there is no further supporting evidence for this finding (Mobley et al., 1988; Wion et al., 1988; Manson et al., 1992). Constitutive expression of the PrP gene in the adult brain suggests that PrP may be critical to neural function in the mammalian central nervous sytsem (Manson et al., 1992). The increase in PrP mRNA with brain development indicates a possible function of PrP^C in brain maturation (Manson et al., 1992).

Although the concentration of PrP mRNA is highest in the CNS neurons, significant levels have also been detected in tissues such as lung and heart (Oesch et al., 1985; Caughey et al., 1988; Robakis et al., 1986; 1996). Robakis et al detected a second 2.4 kb mRNA in adult lung and heart indicating the possibility of different PrP forms in these tissues. Lower amounts are also detected in pancreas, spleen, kidney, liver, and testes (Robakis et al., 1996). Furthermore, PrP is not restricted to neuronal cells. PrP has also been associated with non-neuronal cells such as fibroblasts, lymphocytes, leukocytes, ependymal, meninges, astrocytes, and microglia (Moser et al., 1995; Robakis et al., 1996). The functional role of PrP in such tissues and cells is discussed later.

2.3 The human prion protein (PrP^C) : biosynthesis and structural features

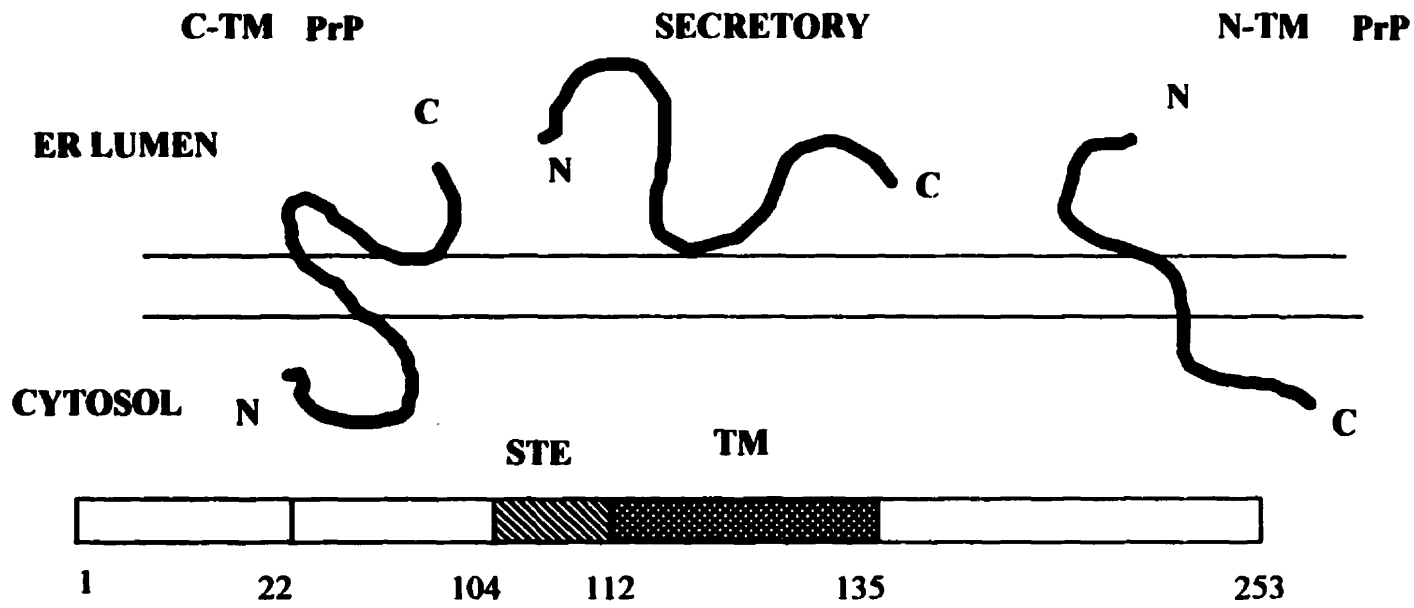
The human prion protein (PrP^C) follows the biosynthetic pathway of other membrane glycoproteins (Cohen et al., 1994; Stahl et al., 1987). PrP^C is synthesized on free ribosomes of the rough endoplasmic reticulum (RER), PrP^C is modified in the Golgi apparatus, and transported to the cell surface, where it is bound by a glycosyl phosphatidyl insositol (GPI) anchor (Bolton et al., 1983; DeFea et al., 1994; Stahl et al., 1987; Borchelt et al., 1990; Caughey and Raymond, 1991). Analogous to other GPI-anchored proteins, PrP^C appears to reenter the cell through a subcellular compartment bounded by cholesterol-rich

detergent insoluble membranes such as caveolae and rafts (Taraboulos et al., 1993; 1995; Shyng et al., 1994; Shyng et al., 1995; Vei et al., 1996; reviewed by Prusiner, 1996; Naslavsky et al, 1997). Structural features of PrP^C (as shown figure 3) include three hydrophobic domains: an NH_2-terminal domain containing a 22 amino acid signal peptide which is cleaved upon entry in the endoplasmic reticulum (ER), a transmembrane domain (amino acids 112-143) almost perfectly conserved (~90%) in a variety of mammalian species, and a COOH-terminal domain consisting of 23 hydrophobic amino acids (as depicted in figure) (Liao et al., 1986; DeFea et al., 1994). One unusual feature of PrP^C is a series of four glycine-rich (Trp-Gly-Gln-Pro-His-Gly-Gly-Ser/Gly) tandemly repeating octapeptide units followed by a nonapeptide unit with an additional amino acid between amino acids 51-91 in the N-terminus (Liao et al., 1986; Puckett et al., 1991). The function of the octapeptide repeats is unknown. Similar repeats have been detected in the amino terminal region of human keratin and and type I collagen (Marchuk et al., 1984; Piez et al., 1984). The octapeptide repeats in human keratin and collagen are implicated in β-sheet formation and assembly of filaments (Locht et al., 1986; Marchuk et al., 1984; Piez et al., 1984). By analogy, the octapeptide repeats of PrP^C may be responsible for promoting the polymerization of the protein into rods and amyloid fibrils as seen in the brains of individuals afflicted with prion diseases (Locht et al., 1986; Hornemann and Glocksherber, 1996).The transmembrane domain (amino acids 112-143) of PrP^C along with a hydrophylic stop transfer effector sequence (STE) located upstream of the membrane-spanning domain, determine the membrane topology of PrP^C. According to cell-free translation studies, PrP^C is synthesized in two different topological forms at the ER membrane. One form of PrP^C is fully translocated into the lumen of the ER consistent with the GPI-anchored secretory form of PrP^C. The second form of PrP^C is transmembrane and can exist either as an N-transmembrane protein with the NH_2 terminus in the ER lumen or C-transmembrane with the

C-terminus in the ER lumen. The rest of the PrP^C protein is exposed to the cytoplasm (refer to figure 4). Mutations in the transmembrane domain or the STE sequence of PrP^C results in higher expression of the C-transmembrane form of the protein in comparison to the other topological forms (Hegde et al., 1998). Overexpression of the C-transmembrane form of PrP^C produces neurodegenerative changes in mice similar to those seen in genetic prion diseases. Also, examination of brains extracted from individuals afflicted with GSS (A117V PrP mutation) revealed an accumulation of C-transmembrane PrP^C rather than PrP^{SC}. The C-transmembrane PrP^C is linked to the neurodegeneration underlying prion diseases such as GSS and maybe others not yet identified. Collectively these findings suggest that any alteration such as dysregulation in the biogenesis and metabolism of PrP^C could result in the expression of one form of PrP^C over others and result in neurodegenerative disease. (DeFea et al., 1994; Hedge etal., 1998). Mutations in PrP^C result in predominant expression of the C-transmembrane PrP^C protein which upon accumulation, results in neurodegeneration.

2.3.1 Post- translational modifications of the PrP^C protein

Post-translational modifications of the PrP^C protein include disulfide bond formation (between cysteine residues 179 and 214), two N-glycosylation sites of Asparagine-X-Threonine (X being Phenylalanine or Isoleucine in PrP^C) at positions 181 and 197, proteolytic processing of PrP^C into N-terminal fragments (8.5-13.5 kDa), and proteolytic cleavage of the last 23 amino acid residues to attach the glycosyl phosphatidyl inositol (GPI) moeity on Ser 231 of the C-terminus. The GPI moeity anchors PrP^C to neuronal cell membranes (Oesch et al., 1985; Liao et al., 1986; Pucket etal., 1991; Stahl and Prusiner, 1991). Heterogeneity of PrP^C in terms of glycosylation patterns is illustrated by a range of bands between 30-35 KDa on western blots (Rogers et al, 1990).



(Hedge et al., (1998), Science 279, pp. 827-833)

Figure 4. - Membrane Topology of PrP^C

2.3.2 PrP^{C} and PrP^{SC} isoforms

Although there is no alternative splicing of the PrP gene, its gene product exists in two different isoforms. The PrP isoforms (PrP^{C} and PrP^{SC}) differ with respect to their synthesis, degradation, structural properties and biochemical properties (Basler et al., 1986; reviewed by Stahl and Prusiner, 1991). Pulse-chase experiments in normal and scrapie-infected murine neuroblastoma cell lines, revealed that PrP^{C} is synthesized and degraded rapidly with a six hour turnover whereas PrP^{SC} shows no sign of turnover (reviewed by Stahl and Prusiner, 1991; Caughey and Raymond, 1991; reviewed by Caughey,1994). It is proposed that the PrP^{C} to PrP^{SC} conversion takes place after PrP^{C} has reached the cell surface, within an endocytic pathway leading to the lysosomes or in lysosomes themselves since that is where PrP^{SC} accumulates (reviewed by Stahl and Prusiner, 1991; Caughey, 1991; reviewed by Caughey, 1994). No differences between PrP^{C} and PrP^{SC} have been noted at the mRNA level or their primary amino acid sequence. However, conformational differences between PrP^{C} and PrP^{SC} exist. According to Fournier transform infrared and circular dichroism spectroscopy studies, the mammalian PrP^{C} molecule has a 43% α-helical, 3% β-sheet conformation and PrP^{SC} possesses a 43% β-sheet pleated misfolded structure and 34% α-helix structure (Pan et al., 1993; Huang et al., 1994). The 43% α-helical content of PrP^{C} is due to the presence of four α-helical domains in segment 108-128 of the protein (Huang et al., 1994). Mutations asssociated with inherited forms of prion diseases are clustered within these regions. Insertion of incorrect amino acids within the α-helical bundles, destabilizes the α-helix structure and promotes the conversion into β-sheets (Prusiner, 1994; reviewed by Prusiner, 1996).

Biochemical differences between PrP^{C} and PrP^{SC} are outlined in the following table:

Table 1: Biochemical properties of PrP^C versus PrP^{SC}

NORMAL PrP (PrP^C)	SCRAPIE PrP (PrP^{SC})
protease K sensitive	27-30 kDa protease K resistant core
detergent soluble	detergent insoluble
anchored to cell membranes via GPI moeity	tighter association with cell membranes
no fibril or plaque formation	accumulates in cells, fibril and amyloid plaque formation

(Lehmann and Harris, 1996).

PrP^C is soluble in denaturing detergents such as Triton X-100 whereas its scrapie isoform is insoluble (Lehmann and Harris, 1996).). Another biochemical property and an important criteria distinguishing PrP^C from PrP^{SC} is protease digestion (McKinley et al., 1983). PrP^C is protease K sensitive whereas PrP^{SC} possesses a 27-30 kDa protease resistant core (McKinley et al., 1983). The 27-30 kDa protease resistant fragment of PrP^{SC} is the same fraction isolated from the brains of animals afflicted with prion disease implying that PrP^{SC} is the infectious agent (Oesch et al., 1985; reviewed by Prusiner, 1996). Also, the association of the two prion isoforms with cellular membranes is different. Based on assays done using the phosphatidylinositol phospholipase C (PIPLC) enzyme to cleave the GPI anchor of the prion protein, Lehmann and Harris reported that PrP^{SC} has a tighter association with cell membranes. PrP^C was released from both normal and scrapie-infected neuroblastoma cells when cleaved with PIPLC but PrP^{SC} remained anchored to the cell membranes. PrP^{SC} may be interacting with other cellular components on the cell surface allowing it to remain tightly bound to the cell (Hay et al., 1987; Borchelt et al., 1990; Stahl and Prusiner, 1990; Lehmann and Harris, 1996). In sum, all features of PrP^{SC} account for its presence and involvement in prion diseases.

2.3.2.1. The existence of prion strains

Distinct strains of prion, although encoded by the same precursor polypeptide chain, and composed of the same amino acid sequence, have been identified on the basis of their different biological, chemical, and physical properties (reviewed by Horwich and Weissmann, 1997; reviewed by Prusiner, 1996). Prion strains display: 1) different sensitivities to protease digestion resulting in distinct patterns of proteolytic cleavage, 2) differential patterns of neuropathological targetting resulting in distinct distribution of lesions, as well as 3) different incubation times in animals prior to experimental transmission. Bruce and collaborators showed that the properties listed above remain unaltered when PrP strains are serially passaged within the same inbred mouse line (Bruce et al., 1991; Carlson et al., 1994; DeArmond et al., 1994; Telling et al., 1996).

The molecular basis for prion strains remains enigmatic. Virologists argue that only conventional pathogens consisting of nucleic acid genome can exist as multiple strains. However, no viral nucleic acid has been discovered to be associated with prions and prion diseases to date. According to the "protein only" hypothesis, there exist structurally distinct PrP^{C} molecules, each of which is converted to abnormal scrapie molecules which further propagate (Bessen et al., 1995). Multiple conformations of the PrP^{C} molecules can account for the selective neuronal targetting in different regions of the brain associated with distinct clinical and pathological manifestations of prion diseases (as defined previously in this discussion). In support of this notion, is a study of two distinct strains of transmissible mink encephalopathy prions designated as hyper (HY) and drowsy (DY). Upon serial passage in hamsters, HY and DY prion strains are distinguished by the different physicochemical properties of the PrP^{SC} accumulated in the CNS of the hamsters. For example, DY is more protease sensitive than HY and displays a different pattern of proteolytic cleavage when subjected on polyacrylamide gel

electrophoresis. Differences in the N-terminus of the two strain types implying distinct conformers account for this finding. Furthermore, the concept of prion strains involving different conformers is supported by the different CJD phenotypes that have been recently linked to different PrP^{SC} glycoforms (reviewed by Collinge, 1997).

2.4 Postulated functions of PrP^{C}

Despite the scientific data accumulated to date which indicates that the expression of PrP^{C} is essential in the development of prion diseases, the normal cellular function of PrP^{C} and the mechanisms controlling its expression remain unclear (Bueler et al, 1992; 1993). However several reports suggest that PrP^{C} may be implicated in the control of lymphocyte proliferation (Cashman et al., 1990), normal synaptic function in mice (Collinge et al., 1994), astrocyte proliferation (Forloni et al., 1993; 1994, leukocyte activation (Diomedes et al., 1996), and cell signalling (Cashman et al., 1990).

PrP^{C} is localized on the surface of normal human B- and T-lymphocytes and lymphoid cell lines (Cashman et al.,1990). Cashman et al demonstrated that polyclonal antibodies to PrP^{C} suppress mitogen-induced activation of lymphocytes. Based on such findings, it was proposed that PrP^{C} participates in lymphocyte activation.

PrP^{C} also plays a role in the normal synaptic function in mice and control of circadian activity. Surprisingly, mice devoid of the prion protein gene are viable. No deleterious effects on the development, behaviour, learning abilities, and life span of the animals have been reported (Bueler et al., 1992; Brenner et al., 1992; DeArmond et al. 1994; Prusiner, 1993). The findings in the PrP^{C} knockout mice suggest that the activity of PrP^{C} is dispensable or compensated by other macromolecules upon its loss (Bueler et al., 1992; Brenner et al., 1992; DeArmond et al., 1994; Collinge et al., 1994). However, abnormalities of GABA-mediated inhibitory neurotransmission and weakened long-term potentiation were reported

in the hippocampus of aged PrP^C-null mice by one research group (Collinge et al., 1994). Loss of PrP^C may contribute to the early synaptic loss, and neuronal degeneration seen in prion diseases (Collinge et al., 1994). In addition, evidence has been provided that PrP^C may be involved in the regulation of sleep intensity or maintaining sleep continuity (Tobler et al., 1996). Loss of PrP^C affects the circadian activity of rhythm and sleep in mice (Tobler et al., 1996). This observation parallels the rhythm and sleep alterations seen in Fatal Familial Insomnia (Tobler et al., 1996; Medori et al., 1992).

An amyloidogenic fragment (amino acids 106-126) of PrP^C is neurotoxic but induces astrocyte proliferation and hypertrophy. In addition, the same peptide caused an increase in the calcium levels in the glial cells. Such findings illustrate the role of PrP^C as a glial growth factor and implicate PrP^C in calcium regulation (Forloni et al., 1993;1994).

Diomedes et al recently demonstrated the activation effects of the prion protein fragment 106-126 in human leukocytes (Diomedes et al, 1996). Synthetic peptide corresponding to residues 106-126 increased the membrane microviscosity, intracellular calcium concentrations, and cell migration in circulating leukocytes. This peptide also increased free radical production in neutrophils and monocytes, stimulating leukocyte migration as a result. Such findings imply a possible role for the immune system in the pathogenesis of prion diseases (Diomedes et al, 1996). However, the clinical signs of prion diseases are confined to the CNS and no immune response accompanies the progression of the illnesses (Berg et al., 1994). Since the 106-126 fragment of PrP is involved in both leukocyte and astrocyte activation, it may represent an important biological active site in the molecule.

In addition, due to its location on the cell surface, PrP^C may be involved in adhesion, transmembrane signalling, or uptake of intracellular ligands (Hope and Manson, 1991). Although not confirmed, PrP^C may be involved in cell-matrix

interactions required for neuron differentiation during development of the CNS (Cashman et al., 1990; Manson et al., 1992). It is suggested that the role of PrP^C as a cell signalling molecule may not be restricted to the CNS, but may have a global impact. PrP^C may act as a cell signalling molecule in peripheral tissues (Cashman et al., 1990; Manson et al., 1992). However the clinical and pathological manifestations of prion diseases do not involve the peripheral nervous sytem (PNS).

In summary, the implications for the role of PrP^C are not exclusive. It is plausible that PrP^C has multiple activities. Disruption of the PrP^C activities by loss of PrP^C or mutations may contribute to the neuronal degeneration and related pathogenic events underlying prion disorders.

3. Homology of the prion protein to Bcl-2 and related proteins

Interestingly, the amino acid sequence of the four octapeptide repeats as well as the nonapeptide repeat in the N-terminal region of the prion protein is highly homologous to the amino acid sequence of the functional and interacting BH2 domain of Bcl-2, Bax, and related proteins. The amino acid sequence of the BH2 domain of Bcl-2 and Bax is not only highly conserved in PrP^C but is repeated four times due to the presence of four octapeptide repeats in the N-terminus of PrP^C. Alignment of the sequences is shown in the following figure:

Figure 5: Alignment of the PrP^C octapeptide repeat and the BH2 domains of Bcl-2 and Bax

PrP^C	G W G Q P H G G
Bcl-2	T W* I Q D N G G
Bax	G W I Q D Q G G

Underlined amino acids are conserved between PrP^C, Bcl-2, and Bax proteins.The (*) symbol indicates that mutations in the tryptophan prevent Bcl-2 from interacting with Bax and abolish Bcl-2's anti-apoptotic activity (Yin et al., 1994). A conversion of the tryptophan to an alanine at codon 188 abrogates Bcl-2's interaction with Bax and cells are no longer protected from apoptosis as a result (Yin et al., 1994). Since Bcl-2 and Bax proteins are implicated in regulating apoptosis, PrP^C may display similar functions. To understand the significance of the homology between the PrP^C, Bcl-2 and Bax and the possible link between them, it is necessary to gain an understanding of the functional aspects of Bcl-2 and its related members.

4. The Bcl-2 gene: structural features

Bcl-2 (B-cell lymphoma leukemia-2) was first identified as a disregulated oncogene at the breakpoint of a t(14:18) translocation occurring in several human follicular lymphomas (Tsujimoto et al., 1984; Tsujimoto et al., 1985). The Bcl-2 gene consists of at least two exons and is transcribed into three overlapping mRNA transcripts: 8.5 Kb, 5.5 Kb, and 3.5 Kb (Tsujimoto et al., 1986; Tanaka et al., 1993). The 5.5 Kb mRNA species gives rise to Bcl-2-α, a 239 amino acid 26 kDa protein and the 3.5 Kb mRNA encodes a 205 amino acid, 22 kDa protein, Bcl-2-β. A significant difference between the Bcl-2-α and Bcl-2-β forms lies at the carboxyl terminus (Tsujimoto et al., 1986; Tanaka et al., 1993; Haldar et al., 1994). The carboxyl terminus of Bcl-2-α contains a hydrophobic stretch of 19 amino acids, followed by two positively charged amino acids (Histidine and Lysine), that allow the post-translational insertion of the Bcl-2 protein to cellular membranes (Tsujimoto et al., 1986; Tanaka et al., 1993; Nguyen et al., 1994;). Bcl-2-β lacks this hydrophobic tail. In addition, Bcl-2-α, being the predominant form of Bcl-2, is present in large amounts in vivo whereas Bcl-2-β is rarely

detected (Tanaka et al., 1993). Therefore, scientific studies have focussed on the 26 kDa form of Bcl-2 (Bcl-2-α).

4.1 Tissue expression and Subcellular distribution of Bcl-2 mRNA

To gain insight on the physiological role of Bcl-2, the distribution of its expression in foetal and adult tissues has been studied. Bcl-2 is expressed in a variety of tissues including those of hematolymphoid, epithelial, neural, endocrine, and mesenchymal type in both adult and foetal stages (Monaghan et al., 1992; LeBrun et al., 1993). In addition, Bcl-2 expression is reported in cells committed to the formation of complex structures such as the glomeruli of kidneys, the glandular epithelium of the skin and intestine, and post-mitotic neurons (Monaghan et al., 1992). Hence, Bcl-2 is topographically restricted to proliferating or long-lived zones in tissues that demonstrate apoptotic cell turnover (Hockenberry et al., 1991).

There is general agreement that the 26 kDa Bcl-2 oncoprotein is intracellular and membrane-bound (Monaghan et al., 1992; Nguyen et al., 1993). Subcellular fractionation assays and conventional immunofluorescence microscopy confirm the residence of Bcl-2 in the nuclear envelope, the endoplasmic reticulum, and the inner and outer mitochondrial membranes (Hockenberry et al., 1991; Monaghan et al., 1992; Krajewsky et al., 1993; Janiak et al., 1994). The pattern of immunostaining seen at the electronmicroscopic level and crude subcellular fractionation assays in lymphoma cells suggest that Bcl-2 is present in a patchy non-uniform manner in the outer mitochondrial membrane as well as within the contact region between the inner and outer mitochondrial membranes. A similar non-uniform patchy distribution of Bcl-2 is noted in the nuclear envelope (Krajewsky et al., 1993). Studies on the distribution of Bcl-2 in the endoplasmic reticulum have not been done in depth. According to studies undertaken by Zhu et al (1996), the subcellular location of Bcl-2 is cell-type dependant and may affect

its anti-apoptotic functions. Both cytosolic and integral membrane forms of Bcl-2 can bind Bax. However, in some cells restricted subcellular localization of Bcl-2 to the endoplasmic reticulum promotes its anti-apoptotic functions than any other location in the cells (Zhu et al., 1996).

4.1.1. Expression of Bcl-2 in the central nervous system (CNS)

Northern blot analysis and in situ hybridization assays reveal a widespread expression of the Bcl-2 gene in the developing and adult central nervous system (CNS) (Ferrer et al., 1994; Castren et al., 1994). The distribution of Bcl-2 mRNA appears to be neuronal with high levels in the neocortex and the hippocampus of the developing rat brain (Castren et al., 1994; Hockenberry et al., 1991; Ferrer et al., 1994). Granule cells of the cerebellum express highest levels of Bcl-2. Glial cells and ependymal cells lining the walls of the ventricles in the striatum are thought to express lower levels of Bcl-2 mRNA (Hockenberry et al., 1991; Ferrer et al., 1994). Expression of Bcl-2 in the CNS is developmentally regulated (Merry et al., 1994). Studies done in the rat brain reveal that Bcl-2 expression is higher in the embryonic stages than in the post-natal period. After birth, Bcl-2 mRNA in the rat brain decreases to a level which remains somewhat steady but further decreases in adulthood. Similar expression patterns of Bcl-2 are thought to occur in the human CNS. The decrease of Bcl-2 levels in the adult CNS correlates wth the amount of neuronal cell death which accompanies the normal development of the CNS (Ferrer et al., 1994; Merry et al, 1994; Veis Novack and Korsemeyer, 1996). In the nervous system, Bcl-2 prevents neuronal cell death induced by serum and glucose deprivation, growth factor withdrawal, calcium ionophore administration, lipid peroxidation, and free-radical exposure (Garcia et al., 1992; Allsopp et al., 1993; Zhong et al., 1993; reviewed by Reed, 1994; reviewed by Wyllie et al., 1993; reviewed by Kroemer et al., 1997; reviewed by Lakshmi and White, 1997). Functional homologues of Bcl-2 such as Bcl-$_{XL}$ are proposed to compensate for

the decrease in Bcl-2 expression in the adult CNS by substituting Bcl-2's functions (reviewed by Reed, 1994).

4.2 Bcl-2 and Bcl-2 related proteins

The structure of the Bcl-2 gene product is depicted in figure 6. Bcl-2 is the prototype molecule of an array of proteins recently identified as the Bcl-2 gene family. The role of the Bcl-2 gene family is to modulate cell death and survival. The Bcl-2 gene family includes both anti-apoptotic as well as pro-apoptotic structural homologs of Bcl-2 ranging from viral species, nematodes, to mammals (Oltvai et al., 1993; Oltvai and Korsemeyer, 1994). Some of the Bcl-2 related proteins are outlined in table 2. Members of the Bcl-2 gene family posess variable degrees of Bcl-2 homology regions: BH1 (amino acids 136-155), BH2 (amino acids 187-202), BH3, and BH4. Bcl-2 homology domains BH1-BH4 allow the Bcl-2 family members to form homo- and heterodimers amongst themselves. Thus, their mechanism of action in regulating cell death and survival is mediated through protein-protein interactions (Oltvai et al., 1993; Oltvai and Korsemeyer, 1994). Indeed, the yeast-two-hybrid system has been used to analyze the interactions among several members of the Bcl-2 family (Sato et al., 1994).

Scientific studies involving Bcl-2 and members of the Bcl-2 family of proteins have allowed a potential breakthrough in understanding the molecular mechanism involved in programmed cell death (apoptosis) of mammalian cells. Apoptosis is a normal physiological process which triggers an intrinsic cell suicide program. Apoptosis involves distinct morphological and biochemical features such as cell shrinking, blebbing of the cell membrane, chromatin condensation, activation of endonucleases, and DNA fragmentation (reviewed by Wyllie, 1987; reviewed by Reed, 1994; reviewed by Kroemer, 1997; reviewed by Lakshmi and White, 1997). Although Bcl-2 prevents both apoptotic and necrotic cell death,

Structural Properties of Bcl-2

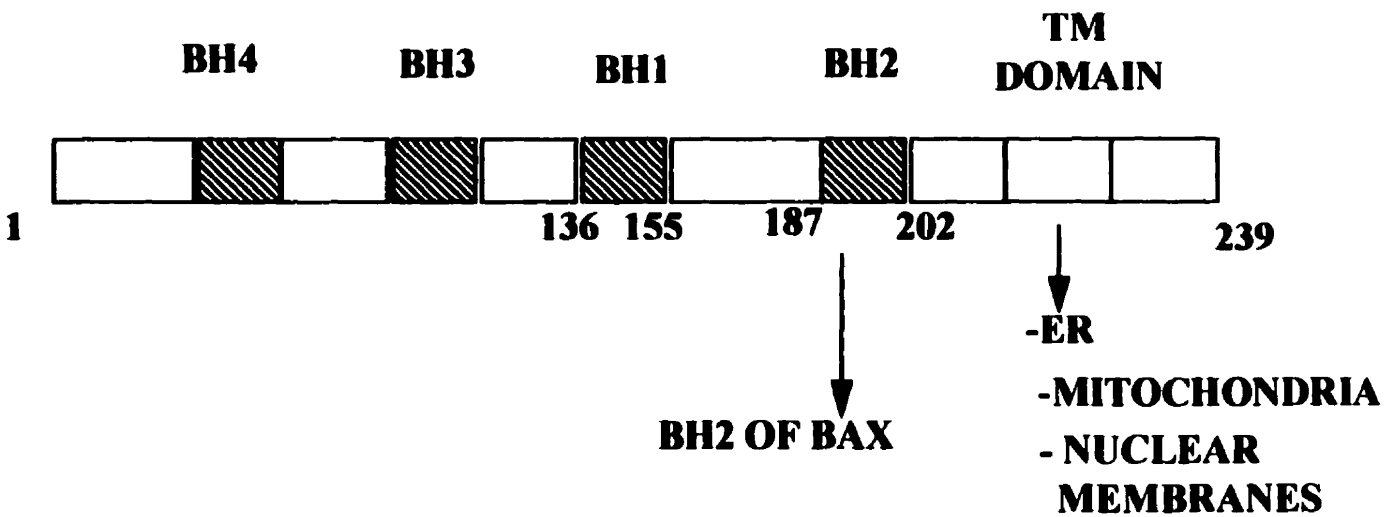


Figure 6 - Structure of the Bcl-2 gene

(Tsujimoto et al., 1987)

Table 2: Bcl-2 Protein Family

ANTI-APOPTOTIC PROTEINS	APOPTOTIC PROTEINS
Bcl-2	Bax
Ced-9 (nematode)	Bad
Mcl-1	Bak
A1	Bik
Bcl-X_L	Bcl-Xs
Bfl-1	Bid
Brag-1	Hrk

Table 2 lists some of the members of the Bcl-2 gene family.

most of the focus has been on its role in modulating apoptosis because of the growing revelance of apoptosis in neurodegenerative disorders.

4.3 Functions of Bcl-2: a regulator of cell death

In 1988, Vaux et al reported that Bcl-2, when overexpressed, prolongs cell survival by blocking apoptosis in various mammalian cells in vivo and in vitro -a notion confirmed by multiple studies (Allsopp et al., 1993; Zhong et al., 1993; reviewed by Wyllie et al., 1993; reviewed by Reed, 1994; Martinou et al., 1994; Farlie et al., 1995; Yang and Korsemeyer, 1996). The exact biochemical mechanisms by which Bcl-2 modulates cell survival and cell death remain unclear but several theories are postulated. These include: 1) inhibition of free radicals and peroxidation, 2) regulation of calcium fluxes, 3) regulation of permeability transition in mitochondria, 4) regulation of cytochrome c release from mitochondria, and 5) protein-protein interactions with Bax (reviewed by Wyllie et al., 1993; reviewed by Reed, 1994; reviewed by McManus and Linnik, 1996; reviewed by Kroemer, 1997; reviewed by Lakshmi and White, 1997).

Apoptotic cell death involves the generation of reactive oxygen species (ROS) and lipid peroxidation (Kane et al., 1993; Hockenberry et al., 1993; Jacobson et al., 1993; 1995;). Bcl-2 may modulate the increase in ROS influenced by the degree of cell death either by decreasing or inhibiting the net cellular generation and accumulation of ROS, or by increasing scavenging of free-radical species .In support of this proposal, is Bcl-2's localization in the endoplasmic reticulum, nuclear envelope, and mitochondrial membranes, all sites involved in ROS production (Hockenberry et al., 1993). Proposed mechanisms accounting for the anti-oxidant effects of Bcl-2 that have been observed include the following: 1) Bcl-2 functions as a direct ROS scavenger, 2) Bcl-2 may possess metal binding properties, and 3) Bcl-2 may inhibit the transfer of electrons from Complex I to

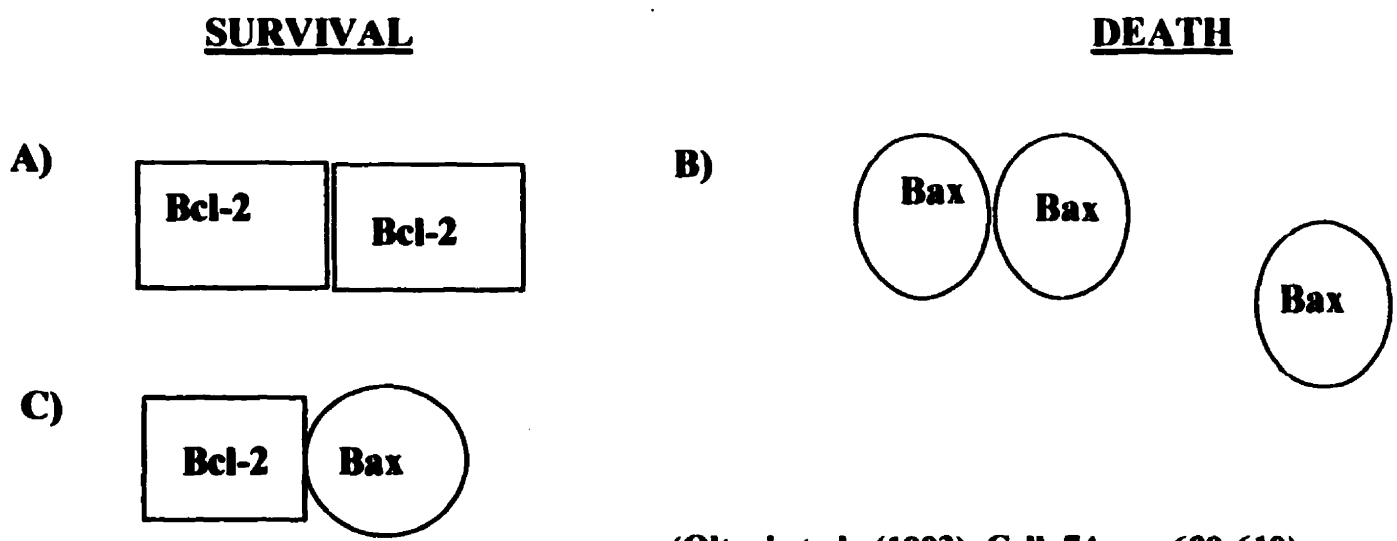
Complex III to oxygen in the inner mitochondrial membrane, blocking the formation of superoxide as a result (Kane et al., 1993; Hockenberry et al., 1993). In addition, Bcl-2 is also able to rescue neural cells that were depleted from glutathione (GSH), a tripeptide involved in protecting cells from oxidative injury (Kane et al., 1993). In situations where oxidative damage is a significant component of cell death ie. in cerebral ischemia, the anti-oxidant effects of Bcl-2 are beneficial (Zhong et al., 1993; Martinou et al., 1994). However, the anti-oxidant effects of Bcl-2 have not been observed in all cellular models of apoptotic death. Jacobson et al reported that Bcl-2's anti-apoptotic functions are carried out even in cells lacking functional mitochondrial DNA (Jacobson et al., 1993; Jacobson et al., 1994; Jacobson et al., 1995). Based on the findings by Jacobson et al, free radical species is one of the events leading to apoptosis that is not necessarily required. Bcl-2 may act at a level upstream to inhibit ROS production and eventually inhibit programmed cell death (Jacobson et al., 1993; Jacobson et al., 1994; Jacobson et al., 1995).

Bcl-2 is also involved in the regulation in calcium fluxes. Calcium is an important second messenger involved in apoptosis. The efflux of calcium from the endoplasmic reticulum into the cytosol of cells is part of a signal transduction pathway resulting in apoptosis (Lam et al., 1994; Martinou et al.,1994; Linnik et al., 1995; Chen et al., 1995; Zhong et al., 1993). Abnormal intracellular concentrations of calcium leads to cellular toxicity and eventually death. Bcl-2, due to its localization in the endoplasmic reticulum, is able to block such events (Lam et al., 1994). In addition, Bcl-2 regulates the calcium fluxes in the mitochondria (Murphy et al., 1996). Under stressful conditions such as hypoxia, ischemia, or treatment of cells with calcium ionophores, cells die due to calcium-induced respiratory injury. Toxic levels of calcium trigger formation of ROS, and as previously mentioned, eventually result in cell death. In the presence of Bcl-2, neural cell mitochondria have a greater resistance to such injury because Bcl-2

enhances their maximal calcium uptake capacity (Murphy et al., 1996). Whether Bcl-2's effect on intracellular calcium homeostasis is direct or indirect remains to be established.

The mechanism of action that received the most focus in the scientific field is Bcl-2's ability to inhibit apoptosis via protein-protein interactions. Members of the Bcl-2 family are able to form homo- and heterodimers amongst themselves and through this mechanism , they act to modulate the fate of a cell (Oltvai et al., 1993; Oltvai and Korsemeyer, 1994). One heterodimerizing partner of Bcl-2 is the 21 kDa cell death protein Bax. Bax shares 21% homology with Bcl-2 (Oltvai et al., 1993). This homology is within the conserved BH1(amino acids 136-155 of Bcl-2) and BH2 (amino acids 187-202 of Bcl-2) domains responsible for both the anti-apoptotic functions of Bcl-2 as well as the formation of Bcl-2-Bax heterodimers (Yin et al., 1994; Sedlak et al., 1995). Evidence supporting this notion is based on the mutagenesis experiments undertaken by Korsemeyer et al (1994). Mutations of the conserved amino acids Trp 188 in the BH2 domain and Gly 145 in the BH1 domain are enough to disrupt the association of Bcl-2 and Bax and eliminate Bcl-2's death repressor activity (Yin et al., 1994). A model has been suggested in which Bcl-2 interacts with Bax and regulates cell survival. As shown in figure 7, overexpression of Bax either as a monomer or homodimer, causes cell death. Bcl-2 is responsible for binding Bax through the BH2 domain, and preventing Bax-Bax homodimers or Bax monomers (Yin et al., 1994) . Bcl-2 rescues the cells from apoptosis as a result. The ratio of Bcl-2 to Bax determines the amount of Bcl-2-Bax heterodimers versus Bax-Bax homodimers and determines the susceptibility of cells to apoptosis (Yin et al., 1994). As soon as the Bcl-2-Bax interaction is disrupted by either mutations or offset in the balance between the Bax-Bax versus Bcl-2-Bax ratios, cells undergo apoptosis (Yin et al., 1994). Other Bcl-2-related proteins like Bcl-$_{XL}$, Bad, Bak etc. are proposed to play similar roles (Sedlak et al., 1995).

Bcl-2 INTERACTS WITH Bax TO PROMOTE CELL SURVIVAL



(Oltvai et al., (1993), Cell 74, pp. 609-619)

Figure 7. - Model explaining Bcl-2-Bax interactions and regulation of apoptosis

Additional functions of Bcl-2 include the regulation of cytochrome c release from mitochondria and the regulation of the transmembrane across the mitochondria (reviewed by McManus et al., 1996). Bcl-2 also acts to inhibit the activity of caspases, key effector molecules of apoptosis which cleave cellular substrates required for cellular survival and maintenance (reviewed by Lakshmi and White, 1997).

4.3.1. Regulation of Bcl-2's anti-apoptotic activity

Although Bcl-2's anti-apoptotic activities are not completely understood, scientific studies indicate that regulation of these activities occurs at the post-translational level. Biochemical and cell biological studies of Bcl-2 activity reveal that Bcl-2's anti-apoptotic activity requires an integral membrane position. The hydrophobic carboxyl terminus of the Bcl-2 protein which allows its post-translational insertion in cellular membranes is necessary for Bcl-2's anti-apototic functions (Nguyen et al., 1993; Nguyen et al., 1994). Constructs lacking this carboxyl domain, or mutations in this domain, reduce Bcl-2's ability to suppress cell death (Nguyen et al., 1994). In addition, post-translational modifications such as phosphorylation or deletion of serine residues of the Bcl-2 protein also have an impact on its ability to regulate apoptosis. Phosphorylation of Bcl-2 inhibits its anti-apoptotic functions while deletion of serine residues promotes Bcl-2's anti-apoptotic activity in neuronal cells (Haldar et al., 1995). Consistent with these findings is evidence that deletion of a negative regulatory loop containing serine and threonine phosphorylation sites in Bcl-2 enables it to promote cell survival (Chang et al., 1997). Recently, Cheng et al., reported that Bcl-2 are substrates for caspases, effector molecules in apoptosis. A loop domain of Bcl-2 (amino acids 32-80) is susceptible to cleavage by caspase 3 (CPP 32). Once cleaved at Asp^{34} of the loop domain, Bcl-2's BH3 domain is activated. The BH3 domain is known to

confer the pro-apoptotic activities of Bax. Bcl-2 is therefore converted to a Bax-like death effector by caspases (Cheng et al., 1997).

5. Hypothesis: PrP^C is involved in an interaction with Bcl-2 and Bax proteins

The present study proposes that PrP^C has an association with Bcl-2, Bax, and possibly other Bcl-2 related proteins based on the homology that exists between BH2 and the four tandem octapeptide repeats as well as the nonapeptide of PrP^C (see figure 5). The homology existing between the amino acid sequences of the proteins suggests that they interact with each other. PrP^C through its interaction with Bcl-2 and Bax, may play a role in the regulation of cell survival and death similar to Bcl-2 related proteins. Mutations, overexpresssion, or conformationally abnormal prion could disrupt such protein-protein interactions and offset the balance between the death and survival states of the cell. This may result in familial, sporadic, or iatrogenic prion diseases. This hypothesis constitutes only the beginning to investigate different aspects of PrP^C function.

The aims of this thesis were to investigate the possible interaction of the entire human prion protein with complete human Bcl-2 and Bax in human cell-free systems and in the human CNS. The Bcl-2-PrP^C and Bax- PrP^C interaction was tested by crossslinking and co-immunoprecipitation studies using recombinant human PrP^C and Bcl-2 proteins expressed in the human lymphoid K562 cell line, and proteins extracted from human primary foetal neurons, human foetal and adult brain tissues. In addition, the PrP^C-Bcl-2 interaction was studied in the yeast-two-hybrid matchmaker system. The levels of expression of all three proteins were examined in different ages of the human CNS in order to establish a relationship between the three proteins and the posssible connection to the neurodegeneration seen in prion disease.

II. MATERIALS AND METHODS

A. EXPRESSION OF Bcl-2 AND PrP^C IN K562

1a. Cloning Bcl-2 and PrP^C cDNAs in Cep 4β: The Bcl-2 cDNA was a kind gift from Walter Nishioka and was already subcloned into the Hind III site of the eukaryotic episomal Cep 4β vector (In Vitrogen, ON, Canada). PrP^C DNA was extracted from peripheral blood or frozen brain tissue and was amplified by PCR as described by Medori et al, 1992. The PrP^C DNA was initially subcloned into the Bam HI and Eco RI restriction sites of pBluescript II KS (pBKS; Stratagene, CA, USA) and subsequently subcloned into the Bam HI and Kpn I restriction sites of the Cep 4β vector (In Vitrogen, ON, Canada).

b. Transfection assays: The K562 human erythrolymphoid cell line (ATTC# GMO5372E NIGMS Human Genetic Mutant Cell Repository, NIH) a kind gift from Mark Tyckocinski, Case Western University, Cleveland, OH, USA, was used for transfection. Transfections were carried out with 5 μg DNA (Cep4 β-PrP^C, Cep 4β-Bcl-2, and Cep 4β vector alone) per 1 x 10^6 K562 cells and 15 μg lipofectin reagent in serum-free OPTI-MEM (Life Technologies, ON, Canada) media as directed in the manufacturer's protocol (Life Technologies, ON, Canada). After 3 hours, 1 ml OPTI-MEM (Life Technologies, ON, Canada) with 20% bovine calf serum (Hyclone, UT, USA) was added. The cells were grown in RPMI 1640 (Canadian Life Technologies, ON, Canada) containing 10 % bovine calf serum (Hyclone, UT, USA). After expansion of the cells for three days, transfectants were selected with RPMI + 10% serum containing 250 μg/ml Hygromycin β (Boerhinger Mannheim, QC, Canada). All cells were fed with RPMI 1640 + 10 % serum containing 250 μg/ml Hygromycin β every 48 hours. Following a period of approximately two weeks, stable transfectants were observed under the microscope. These were attached on the bottom of the flask as colonies with a bee-hive appearance. Wild-type K562 looked shrivelled and dead

indicating a successful transfection. The term "stable" is used for these transfectants since even upon freezing and thawing, transfectants are still maintained.

c. Extraction of protein from the transfected K562 cells: Transfected K562 cells were collected (about 1 million cells per flask) and centrifuged at 600 xg at 4°C for 5 minutes. The pellets were homogenized in 200 µl 20 mM sodium phosphate buffer pH 7.5. The homogenates were centrifuged at 600 xg for 30 minutes at 4°C and the pellets were rehomogenized in 200 µl 20 mM sodium phosphate buffer pH 7.5. Protein concentration was determined by the BCA (Bicinchinonic acid) protein assay (Smith et al, 1985) following the specifications of the manufacturer (Pierce, IL, USA).

d. Assessing the expression of PrP^C and Bcl-2 in K562 by western blot analysis: Proteins (40 µg) extracted from the Cep 4β-PrP^C, Cep 4β-Bcl-2 and Cep 4β -transfected K562 cells, as well as wild-type K562, were mixed with 3 µl of 4X SDS/Sample loading buffer (300mM Tris-HCl, pH 6.8, 600mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol), and separated in parallel with prestained low molecular weight markers (BioRad, ON, Canada) on 15% SDS-polyacrylamide gels by electrophoresis. Separated proteins were transferred onto Immobilon-P membranes (Millipore, MA, USA). Membranes were blocked in Blotto A (5% (w/v) low fat milk in TBST (Tris-buffered saline; 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween-20) for 1 hour, and immunoblotted with either monoclonal anti-PrP 3F4 antibody (1/1000 dilution in 5% blocking solution; kind gift from Neil Cashman, Montreal Neurological Institute) or monoclonal anti-human Bcl-2 antibody (1/400 dilution in 5% blocking solution; #sc 509; Santa Cruz, CA, USA overnight at 4°. Membranes were washed in TBST buffer (10 mins/wash) three times and then incubated with the appropriate secondary antibody conjuguated to alkaline phosphatase (AP)-goat-anti-mouse-AP (1/500 in

TBST; Jackson Labs, PA, USA) for 2 hours at room temperature. The blots were developed with 20mls alkaline phosphatase buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5mM $MgCl_2$) containing 132 µl NBT(Nitro blue tetrazolium chloride; Fisher, QC, Canada; dissolved in 70% diethylformamide) and 66 µl BCIP (5-bromo-4-chloro-3-indoyl phosphate; Fisher, QC, Canada; dissolved in 100% diethylformamide to detect the immunoreactive proteins.

II B. IN VITRO PrP^C AND Bcl-2 INTERACTIONS

1. Assessing the PrP^C-Bcl-2 interaction in K562 cells

a. Coimmunoprecipitation assay: Total protein extracts (150 µg) from cell lysates generated from K562-Cep 4β-PrP^C and K562-Cep 4β-Bcl-2 transfected cells were immunoprecipitated with polyclonal anti-human Bcl-2 (#sc 493; Santa Cruz, CA, USA) antisera. Immunoprecipitations were carried out in 200 µl total volume consisting of 150 µg protein, 30 µl protein A agarose (Sigma, MO, USA), 50 µl 5X RIPA buffer (10mM Na_2PO_4, 0.15M $NaCl_2$, 15 NP-40, 2mM EDTA, pH 8.0, 0.05% PMSF, 0.1µg/ml pepstatin A, 1µg/ml TLCK, 0.5µg/ml leupeptin (all protease inhibitors were purchased from Sigma, MO, USA) and 5µl of the appropriate antisera. After mixing on a rotator overnight at 4°C, the immunoprecipitations were centrifuged at 16 250 x g at room temperature, and washed in 1X RIPA buffer three times. They were placed in 20 µl 4X SDS/Sample loading buffer (300mM Tris-HCl, pH 6.8, 600mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol), boiled for 5 minutes, and separated on a 15% SDS-polyacrylamide gel by electrophoresis. Western blot analysis was done as described above.

2. Investigating the PrP^C-Bcl-2 and PrP^C-Bax interactions in human brain

a. Preparation of human primary fetal neurons

Human fetal cultures (from embryos of 10-18 weeks of gestation) were prepared by the lab technician as previously described (LeBlanc, 1995).Guidelines

established by MRC, NIH, the Jewish General Hospital and McGill University (Montreal, QC, Canada) were followed.

b. Extraction of protein from human fetal neurons, fetal and adult brains

On the eleventh day of seeding the primary fetal neurons, they are ready for experimentation. Approximately 40 x 10^6 primary human fetal neurons were collected by trypsinization (2.5 % trypsin was used). The resulting cells were washed twice in 1 x PBS buffer containing protease inhibitors : 1 μg/ml TLCK, 0.5 μg/ml Leupeptin, 0.05% PMSF, and 0.1 μg/ml Pepstatin A (all protease inhibitors were purchased from Sigma, MO, USA) and homogenized in 200μl 20 mM sodium phosphate buffer pH 7.5. Extraction of protein was done as described for K562 cell lines. For the brain extracts, frozen fetal (13-15 weeks) or adult (46-91 years) frontal brain tissue was cut and used for protein extraction. Quantitation of proteins was done by BCA assay following the manufacturer's protocol (Pierce, IL, USA).

c. Assessing the expression levels of PrP and Bcl-2 in human primary neurons, human fetal brain, and human adult brain by western blot

Forty micrograms of protein extracts from human fetal neurons, human fetal brain and human adult brain tissues were mixed with 3 μl of 4X SDS/Sample loading buffer (300mM Tris-HCl, pH 6.8, 600mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol), and separated in parallel with prestained low molecular weight markers (BioRad, ON, Canada) on 15% SDS-polyacrylamide gels by electrophoresis. Western blots were performed as described for K562 cells. Monoclonal anti-PrP 3F4 antibody (1/1000 dilution in 5% blocking solution; kind gift from Neil Cashman, Montreal Neurological Institute) or monoclonal anti-human Bcl-2 antibody (1/400 dilution in 5% blocking solution; #sc 509; Santa Cruz, CA, USA) were used for immunoblotting.

d. Investigating the PrP-Bcl-2 and PrP-Bax interaction in human fetal and adult brain by co- immunoprecipitation and western blotting

Total protein extracts (150 μg) from fetal and adult brains were immunoprecipitated with either polyclonal anti-human Bcl-2 (#sc 493, Santa Cruz, CA, USA) , polyclonal anti-human PrP R155 antisera (detects amino acids 56-92 of the N terminus of PrP; made in our lab) or polyclonal anti-human Bax antisera (#sc 509, Santa Cruz, CA, USA). Immunoprecipitations were carried out in a volume of 200 μl consisting of the 150 μg protein extract , 30 μl protein A agarose (Sigma, MA, USA), 50 μl 5 X RIPA buffer (10mM sodium phosphate, 0.15 M sodium chloride, 1% NP-40, 2mM EDTA pH 8, 0.05% PMSF, 0.1 μg/ml pepstatin A, 1 μg/ml TLCK, 0.5 μg/ml leupeptin) and the appropriate antisera (5μl). Immunoprecipitates were subjected to western blot analysis as described above. Monoclonal anti-PrP 3F4 antibody (1/1000 dilution in 5% blocking solution), monoclonal Bcl-2 antibody (1/400 dilution in 5% blocking solution), or monoclonal Bax (1/5000 dilution in 5% blocking solution; YTH -2D2; Trevigen, USA) were used for immunoblotting. Competition experiments were also performed by adding 1 μg of the appropriate peptide to the immunoprecipitation mix. Synthetic peptides against anti-human PrP R155, polyclonal Bcl-2 (#sc 492P; Santa Cruz, CA, USA), and Bax (#sc 493P, Santa Cruz, CA, USA) were used. Blots were also reprobed with monoclonal Bcl-2 and monoclonal Bax antibodies. Following the incubation with the primary antibodies, the blots were washed three times with TBS-T (Tris-buffered saline; 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% Tween-20) and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP)-goat anti-rabbit-HRP (1/5000 in Blotto A) or goat anti-mouse-HRP (1/1000 in Blotto A) (both from Santa Cruz, CA, USA) for 1 hour at room temperature. Immunoreactive proteins were detected with ECL Western Blot Detection Reagents (Amersham, ON, Canada) according to the manufacturer's specifications.

e. Adsorption experiments

Adsorption experiments were done to test the specificity of both the polyclonal R155 and the polyclonal Bcl-2 antisera. In one milliliter Blotto A (5% (w/v) low fat milk in TBST, 5 µl R155 antisera with 1 µg synthetic peptide against R155 were added. In a separate milliliter Blotto A, R155 antisera alone was added. The mixes were incubated on the rotator overnight at 4°C, centrifuged at 16 250 x g at room temperature for 5 minutes and the supernatants were used for immunoblotting of membranes containing 40µg proteins from human fetal and human adult brain. Adsorption reactions for bcl-2 antibodies were done using synthetic peptide against the anti-human Bcl-2 antisera (1/400 dilution in 5% blocking solution; #sc 492P; Santa Cruz, CA, USA) following the same procedure described above.

II C. IN VIVO Bcl-2 - PrP^C INTERACTIONS

1. The yeast-two-hybrid matchmaker system

a. Cloning of PrP and Bcl-2 in pGAD424 and pGBT9 vectors

Using Bam HI and Eco RI restriction enzymes, PrP was released from the pBluescript vector (Stratagene, ON, Canada) and was then subcloned into the Bam HI/ Sal I sites of pGBT9. Pvu II and Bam HI restriction enzymes were used to release Bcl-2 from the Cep 4β vector. Bcl-2 was then subcloned into the Bam HI/ Pvu II sites of pGAD424. Cloning procedures were done following guidelines from Sambrook et al, 1989..

b. Characterization and co-transformation of YRG-2 yeast

Saccharomyces cerevisiae yeast strain YRG 2 (Stratagene, ON, Canada) carries mutations which ensure that the endogenous GAL 4 gene is not expressed. YRG-2 contain three auxotrophic markers: 1) tryptophan (trp 1), 2) leucine (leu 2) and 3) histidine (his 3) as well as two reporter genes: His-3 encoding histidine and Lac Z encoding β-galactosidase. The YRG-2 cells were grown in YPD (20 g/L

Difcopeptone, 10 g/L yeast extract, 2% glucose) media overnight at 30°C till late-log phase (1 x 10^7 cells). Cells were centrifuged at 600 x g at 4°C for I5 minutes, washed in sterile water three times, and resuspended in 1M sorbitol (Fisher, QC, Canada) and 1M dithiothreitol (DTT) (Boerhinger Mannheim, QC, Canada). One microgram DNA: Bcl-2-pGBT9, PrP-pGAD424, both constructs, or vectors alone, was mixed with 0.8 mls competent yeast cells and transformed by electroporation at 1.5 Kilovolts, 25 μF capacitance and 200 Ω in resistance with the Bio-Rad gene pulser .

c. Isolation of yeast co-transformants:

Synthetic dropout (SD) medium is used for selection of yeast containing a plasmid. Cells were plated out on synthetic dropout media (SD) (6.7 g/l Difco yeast nitrogen base without amino acids, 182.2 g/l D-sorbitol, 40mls/l 50% glucose and 100mls/l dropout solution (300 mg/l L-isoleucine, 1500 mg/l L-valine, 200 mg/l L-adenine hemisulfate salt, 200 mg/l L-arginine HCl, 200 mg/l L-histidine HCl monohydrate, 1000 mg/l L-leucine, 300 mg/l L-lysine HCl, 200 mg/l L-methionine, 500 mg/l L-phenylalanine, 2000 mg/l L-threonine, 200 mg/l L-tryptophan, 300 mg/l L-tyrosine, 200 mg/l L-uracil)) selection agar plates lacking tryptophan, leucine, tryptophan and leucine, or histidine, tryptophan, and leucine amino acids. Plates were incubated at 30°C for 4 days and cotransformants were selected.

d. β-galactosidase liquid culture assay:

The β-galactosidase liquid culture assay was done following the protocol from Stratagene with certain modifications: yeast cotransformants were grown in 2 mls YPD media at 30° till mid-log phase (OD 600nm- 0.5-0.8). 0.1 mls culture were used for this assay. Cultures were washed in Z buffer (60mM Na_2HPO_4, 40mM NaH_2 PO_4 , 10mM Mg_2SO_4) containing β-mercaptoethanol. Pellets were resuspended in 0.7mls Z buffer. To each sample 500μl chloroform and 500μl 0.1% sodium dodecyl sulfate, 160μl of 4mg/ml o-nitrophenyl-β-galactoside / Z buffer

was added ,and were incubated at 30° for 1 hour. To quench the reactions, 0.4mls of 1M sodium carbonate was added. Product formation was determined by reading the OD at 420nm. β-galactosidase units were calculated using the following formula: β-gal units: 1000x [OD_{420}/t x V x OD_{600}] where OD=absorbance, t=60 mins of incubation and V=0.1mls culture used for this assay. Average β-galactosidase units were calculated from the three independant clones used for this assay. Results were standardized to the transfection done in the absence of DNA.

e. Colorimetric filter assay for β-galactosidase:

Cotransformants were streaked on a circular whatman filter paper and placed on the surface of the appropriate SD agar plates (SD –His,–Trp,–Leu, and SD +His,–Trp,–Leu). Plates were incubated at 30°C for 4 days. The filter was lifted and placed on dry ice colonies facing up till it was uniformly frozen. Once thawed, the filter was placed on a clean whatman filter presoaked in Z buffer /X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside)/ β-mercaptoethanol solution (100mls Z buffer, 0.27 mls β-mercaptoethanol, 1.67 mls X-gal). Filters were incubated at 30°C and checked periodically for the appearance of a blue colour, an indicator of β-galactosidase activity. Four hours was the average period of time elapsed between the filter assay and the appearance of blue colour for our assays.

2. Detection of Bcl-2-PrP and Bax-PrP proteins by in vivo crosslinking:

To further confirm the interactions of Bcl-2 and PrP^C as well as PrP^C and Bax in vivo, crosslinking experiments were performed. Human primary fetal neurons known to expresss high levels of PrP^C and Bcl-2 were used for in vivo crosslinking experiments. Cells were treated with DSP (Dithiobis-succinimidyl proprionate) at various concentrations 0 mM, 1 mM, 2 mM, 4 mM as described by the manufacturer (Pierce,IL,USA). Proteins were collected in NP 40 lysis buffer (100-200 µl). An equal volume of each lysis product was immunoprecipitated with either anti-PrP R155 polyclonal antisera or anti-human monoclonal anti-human PrP

3F4 and separated on a 15 % polyacrylamide gel under non-reducing conditions. Anti-PrP 3F4 , anti-Bcl-2 and anti-Bax antibodies were used for immunoblotting.

IID. Determination of the expression levels of PrP, Bcl-2, and Bax in the cerebellum :

Frozen cerebellum tissue was obtained from both human adult brain (46 year old, 80 year old, 91 year old individuals) as well as human fetal brain (13 weeks old). The post-mortem brain tissues were kindly collected by Dr. Stephen Albrecht (Jewish General Hospital), and Dr. Cynthia Goodyer (Montreal Children's Hospital). A small piece of tissue was cut from the original frozen samples and homogenized in 200 µl 20mM sodium phosphate buffer pH 7.5. Extraction of proteins was carried out following the same procedures as described previously. Forty micrograms of protein were separated by 10% SDS-PAGE. Western blot analysis using anti-human monoclonal Bcl-2, PrP (3F4), and Bax antibodies was done as previously described.

III Results

1. IN VITRO INTERACTION OF PrP^C AND Bcl-2

1.1 Expression of Bcl-2 and PrP^C in the K562 human lymphoid cells

The human K562 erythrolymphoid cell line was chosen to express the Cep 4β-PrP^C and Cep 4β-Bcl-2 DNAs respectively. The advantages of using the K562 cell line and the eukaryotic episomal Cep 4β vector over other systems are numerous: 1) K562 cells can be easily grown in culture, 2) wild-type K562 cells do not express high levels of endogenous PrP^C or Bcl-2, 3) K562 cells are easy to transfect using lipofectin reagent, 4) the post-translational and conformational modifications of the PrP^C and Bcl-2 proteins in K562 cells are representative of what occurs in a human eukaryotic cell, 5) the inserted PrP^C and Bcl-2 genes are under the control of a Cytomegalovirus (CMV) promoter and Cep 4β can replicate to attain many copies per cell nucleus resulting in high levels of expression of the cloned genes of interest, and 6) Cep 4β possesses a hygromycin B resistance marker allowing the selection of transfected cells within a period of two weeks. Transfectants, when observed under the microscope, are attached to the bottom of the culture flask, form colonies with a bee-hive appearance and expand quickly. Wild-type K562 cells look shrivelled and die after treatment with hygromycin B (250 μg/ml) antibiotic.

According to figure 8A, the K562 cells were successfully transfected with the Cep 4β-PrP^C (designated PrP) and Cep 4β-Bcl-2 (designated Bcl-2) constructs since transfected K562 cells are expressing high levels of Bcl-2 and PrP^C in comparison to control wild-type. Transfected K562- Cep 4β-PrP^C cells show high levels of the expected series of PrP^C proteins between 25-36 kDa. The multiple bands seen on the western blot represent alternate forms of the different post-translationally modified PrP^C such as glycosylation. This is an example of the heterogeneity of the PrP^C molecule. Transfected K562- Cep 4β-Bcl-2 cells are

expressing high levels of Bcl-2. A sharp 26 kDa immunoreactive band corresponding to Bcl-2 is seen in the transfected K562 cells in comparison to the wild-type K562 cells.

Once it was confirmed by western blot analysis that the K562 cells were transfected with Bcl-2 and PrP^C, the next step was to test a possible PrP^C-Bcl-2 interaction by co-immunoprecipitation. Proteins (150 μg) extracted from K562 Cep 4β-PrP^C (designated as PrP in figure 8B) and K562 Cep 4β-Bcl-2 (designated as Bcl-2 in figure 8B) transfected cells were mixed (designated as PrP x Bcl-2 in figure 8B) and immunoprecipitated with polyclonal anti-human Bcl-2 antisera. Samples were separated on a 10% acrylamide gel. Monoclonal 3F4 anti-human PrP antibody shows the co-immunoprecipitated bands of PrP^C present in the PrP^C x Bcl-2 mix. Monoclonal anti-human Bcl-2 antibody shows the sharp 26 kDa Bcl-2. Results in figure 8B show that polyclonal anti-human Bcl-2 antisera immunoprecipitates both Bcl-2 and PrP^C, and all isoforms of PrP^C such as present in the non-immunoprecipitated control, bind Bcl-2. The second Bcl-2 immunoreactive band (above the expected 26 kDa Bcl-2) may be the result of a post-translationally modified Bcl-2. There is evidence suggesting that Bcl-2 is post-translationally modified by phosphorylation on serine and threonine residues. Phosphorylation inhibits Bcl-2's anti-apoptotic activity (Haldar et al, 1995).

Figure 8. Western blot analysis of PrP^C and Bcl-2 expression and co-immunoprecipitation in K562 cells

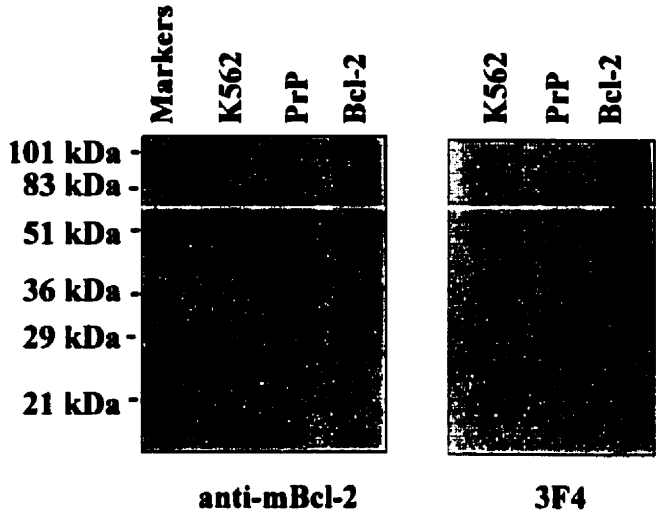
A. Expression of PrP^C and Bcl-2 in K562 cells

Each lane represents forty micrograms of protein extracted from wild-type K562 **(K562)**, K562 transfected with Cep 4β-PrP **(PrP)**, K562 transfected with Cep 4β-Bcl-2 **(Bcl-2).** Proteins were separated on an SDS polyacrylamide gel and submitted to western blotting. Monoclonal anti-human PrP **(3F4)** and monoclonal anti-human Bcl-2 **(anti-mBcl-2)** antibodies were used for immunodetection. A sharp band at 26 kDa representing Bcl-2 and multiple bands between 25-45 kDa representing PrP^C are highly expressed in transfected K562 in comparison to wild-type. Molecular weight markers are indicated in kDa.

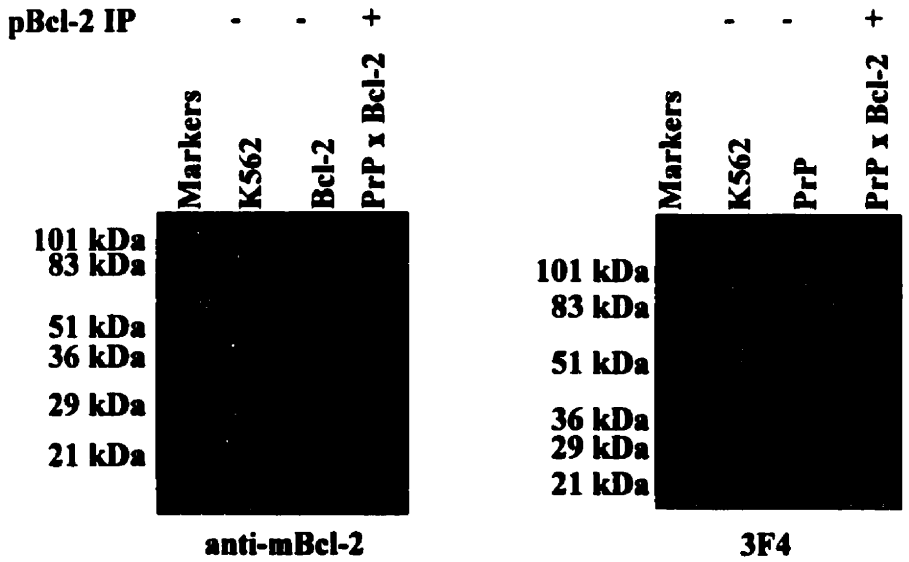
B. Co-immunoprecipitation of Bcl-2 with PrP^C in K562 using anti-human Bcl-2 antisera.

Proteins (150 μg) extracted from PrP-transfected K562 **(PrP)** and Bcl-2-transfected K562 **(Bcl-2)** were mixed **(PrP x Bcl-2)** and immunoprecipitated with polyclonal anti-human Bcl-2 antisera **(pBcl-2 IP +)**. Immunoprecipitates and 40 μg of non-immunoprecipitated extracts **(pBcl-2 IP -)** were separated on a 10% SDS polyacrylamide gel. Bcl-2 and PrP^C were detected as described in 8A. Polyclonal Bcl-2 antisera immunoprecipitates both Bcl-2 and PrP^C. All isoforms of PrP^C (25-45 kDa), detected in the non-immunoprecipitated control **(pBcl-2 IP -)**, co-immunoprecipitate with Bcl-2.

A.


Markers
K562
PrP
Bcl-2
101 kDa
83 kDa
51 kDa
36 kDa
29 kDa
21 kDa
anti-mBcl-2
K562
PrP
Bcl-2
3F4

B.


pBcl-2 IP
- - +
Markers
K562
Bcl-2
PrP x Bcl-2
101 kDa
83 kDa
51 kDa
36 kDa
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anti-mBcl-2
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PrP
PrP x Bcl-2
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29 kDa
21 kDa
3F4

1.2. In vitro interaction of PrP^C and Bcl-2 in human brain

Until now, the systems used to study the expression of PrP^C and Bcl-2 consist of rodent primary neuron cultures. Although such systems have allowed elucidation of the molecular genetics and possible roles of PrP and Bcl-2 proteins, they are not entirely relevant to the human neurons. Since Bcl-2 and PrP^C proteins are highly expressed in the human brain, highly purified primary human foetal neurons were established for the study of PrP^C and Bcl-2 expression as well as their interaction. In addition to the primary human foetal neurons, frozen post-mortem tissue from human foetal brain as well as human adult brain was used to investigate both the expression of PrP^C and Bcl-2 as well as the PrP^C-Bcl-2 interaction. Figure 9A shows the different levels of expression of PrP^C and Bcl-2 in foetal neurons, foetal brain, and adult brain. Forty micrograms of protein extracted from primary human foetal neurons, foetal and adult brain, were separated on a 10% SDS-PAGE and immunoblotted with monoclonal anti-human Bcl-2 and PrP antibodies respectively. As seen in mice brain (Merry et al., 1994), Bcl-2 expression is more intense in the human foetal brain than in the human adult brain. In contrast, PrP^C levels of expression are higher in the adult brain than in foetal brain. The pattern of bands detected for PrP^C (between 25-34 kDa) in the adult brain differs from the pattern of bands seen in foetal brain. Additional bands present below 16 kDa at a stronger intensity in the adult brain may represent the proteolytically cleaved N-terminal fragments (8.5-13.5 kDa) of PrP^C. Human foetal neurons display a pattern similar to the adult brain with respect to both PrP^C and Bcl-2 expression.

Once the levels of expression for the PrP^C and Bcl-2 proteins were assessed in both human foetal and adult brain, the PrP^C-Bcl-2 interaction was studied. Figure 9B shows a western blot analysis of the co-immunoprecipitation of PrP^C and Bcl-2 in human foetal brain. Total protein extracts (150μg) were immunoprecipitated with either polyclonal anti-PrP (R155) antisera which detects

amino acids 56-92 of the N-terminus of PrP^C or polyclonal anti-human Bcl-2 antisera. The immunoprecipitates were separated on a 15% SDS-PAGE acrylamide gel and immunoblotted with monoclonal anti-human antibodies 3F4 or Bcl-2. When proteins extracted from human foetal brain were immunoprecipitated with polyclonal Bcl-2 antisera and immunoblotted with monoclonal anti-PrP antibody (3F4), PrP^C (thick sharp band at 34 kDa) was detected (lane 3 of left panel, figure 9B). Also, when proteins were immunoprecipitated with polyclonal anti-PrP (R155) antisera and immunoblotted with monoclonal Bcl-2 antibody, a 26 kDa band corresponding to Bcl-2 was detected (lane 5 of right panel, figure 9B). The same experiments were performed using protein extracts of adult human brain. The adult human brain constitutes an excellent system for studying the PrP^C-Bcl-2 interaction since prion diseases normally manifest in the fourth decade of adult life. Figure 9C shows co-immunoprecipitation of PrP^C and Bcl-2 in the adult human brain of a 46 year old individual. Less Bcl-2 is present in adult brain since Bcl-2 expression decreases with age. The decrease in Bcl-2 expression with age explains why less Bcl-2 is bound to PrP^C as seen in the western blot in figure 9C.

To test the specificity of the antibodies used for the immunoprecipitation experiments, adsorption reactions using specific synthetic peptides against both the R155 and the Bcl-2 antisera were performed. Results are seen in figure 9D. In both foetal and adult human brain, the bands detected by each antibody (- lane) are specifically adsorbed by each peptide (+ lane). To further confirm the PrP^C-Bcl-2 interaction shown by co-immunoprecipitation, additional primary antibody control experiments were done using protein extracted from human foetal brain. Controls include competition with a synthetic peptide against the R155 antisera, immunoprecipitation with pre-immune antisera, and the absence of a first antibody in the immunoprecipitation. Results are shown in figure 9E. In the upper panel of figure 9E, the competition experiment done using 1 μg synthetic peptide against the polyclonal R155 antisera shows that the thick 34 kDa band detected with R155

antisera, is specific for PrP^C since this band is competed out. When proteins were immunoprecipitated with the pre-immune antisera or when no first antibody was used in the immunoprecipitation mix, only background appears, indicating once again that PrP^C is only detected by the R155 antisera. The same primary antibody control experiments were done to test for the specificity of the band detected by the monoclonal Bcl-2 antibody. When 150 μg of proteins extracted from foetal brain were immunoprecipitated with polyclonal R155, immunoreactive proteins representing Bcl-2 (26 kDa), as seen in the non-immunoprecipitated control, appear on the western blot probed with monoclonal Bcl-2. In the presence of the competing peptide against R155 and pre-immune antisera, the appearance of this immunoreactive band is eliminated. The same results when the western blot is not probed with monoclonal Bcl-2 antibody (last lane, lower panel, figure 9E).

On the basis of the results obtained by the co-immunoprecipitation experiments performed in the human K562 erythrolymphoid cell line and the human foetal and adult brain systems, PrP^C and Bcl-2 interact in vitro.

Figure 9. Western blot analysis showing interaction of PrP^C and Bcl-2 proteins in human primary foetal neurons, human foetal and adult brain

A. Expression of PrP^C and Bcl-2 proteins in human primary foetal neurons, human foetal and adult brain

Each lane represents 40 μg of protein extracted from human foetal brain, human adult brain, and primary human foetal neurons. Monoclonal anti-human PrP **(3F4)** and anti-human Bcl-2 **(mBcl-2)** antibodies were used for immunoblotting.

B. Co-immunoprecipitation of PrP^C and Bcl-2 in human foetal brain

The first lane of each panel represents 40 μg non-immunoprecipitated protein from foetal brain. Immunoprecipitation of 150 μg protein with polyclonal anti-human PrP R155 antisera **(R155)** or polyclonal Bcl-2 antisera **(pBcl-2)** were immunoblotted with monoclonal 3F4, or Bcl-2 antibodies. Co-immunoprecipitation of PrP^C and Bcl-2 in human foetal brain suggests an interaction between PrP^C and Bcl-2.

C. Co-immunoprecipitation of PrP^C and Bcl-2 in human adult brain

Same as 9B except the proteins were extracted from human adult brain. PrP^C and Bcl-2 co-immunoprecipitate in human adult brain. Notice that there are lower levels of Bcl-2 in the adult brain. Less Bcl-2 is bound by PrP^C

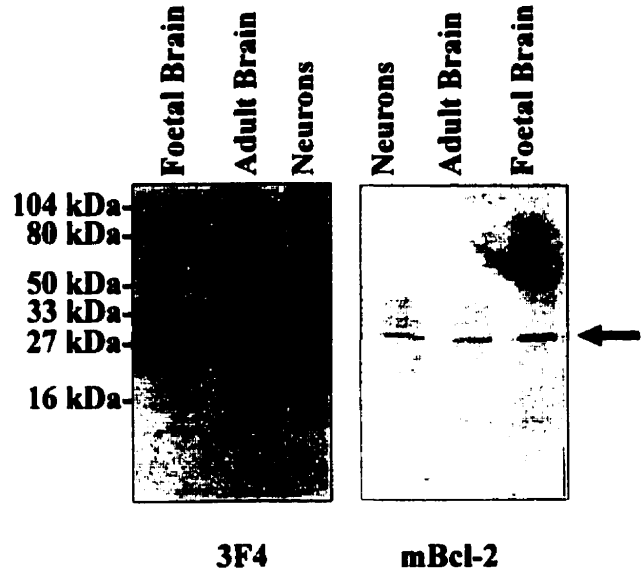
D. Adsorption experiments with synthetic peptides against epitopes recognized by the R155 and Bcl-2 antisera in adult and foetal brain

Forty micrograms of protein extacted from either adult or foetal human brain, immunoblotted with non-adsorbed **(-)** R155 or Bcl-2 antibodies, were separated on an SDS polyacrylamide gel in parallel with 40 μg proteins immunoblotted with R155 or Bcl-2 and 1 μg synthetic peptide against each antisera **(+)**.

E. Western blot analysis showing controls done on one set of co-immunoprecipitation experiments in foetal brain

In the upper panel, the first lane shows 40 μg proteins extracted from human foetal brain non-immunoprecipitated **(non-IP)**. The other lanes show immunoprecipitation of 150 μg proteins with polyclonal anti-human PrP **(IP R155)**, polyclonal R155 antisera competed with a peptide against R155 **(IP R155 + peptide)** or pre-immune antisera **(IP pre-immune)**. The upper panel represents detection of PrP with monoclonal 3F4 antibody. Lane 5 shows 150 μg proteins extracted from human foetal brain immunoprecipitated with polyclonal Bax antisera and no 3F4 antibody used for probing **(no first Ab)**. The lower panel represents the same blot probed with monoclonal Bcl-2.

A.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Adult Brain
Neurons
Neurons
Adult Brain
Foetal Brain
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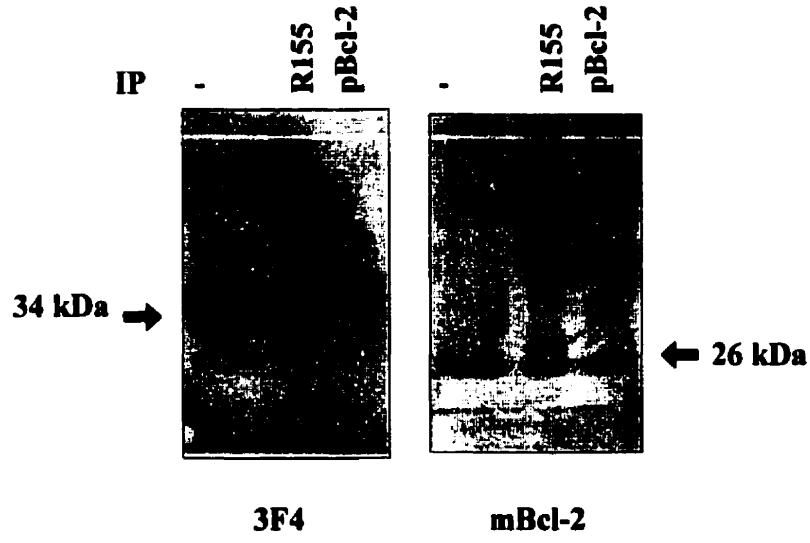
80 kDa
50 kDa
33 kDa
27 kDa
16 kDa
3F4
mBcl-2

B.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34 kDa
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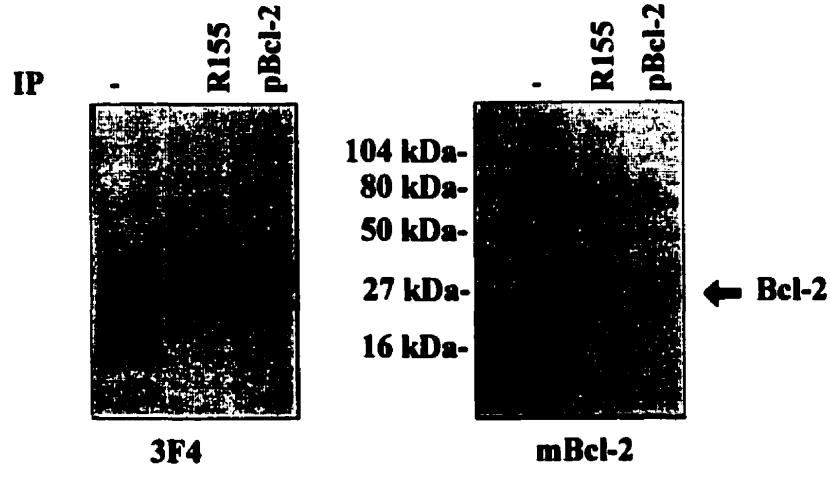
mBcl-2

C.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-
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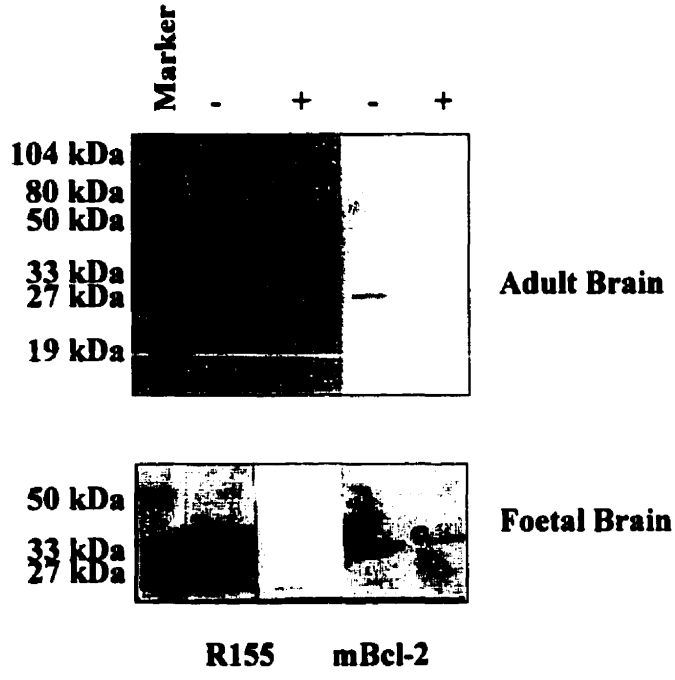
3F4
mBcl-2

D.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+
-
+
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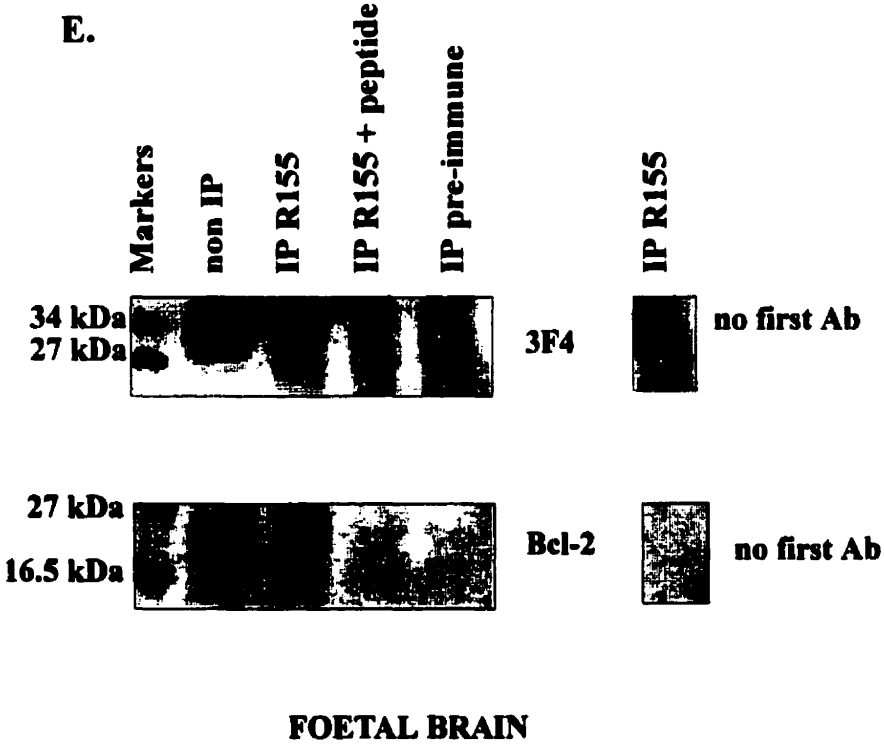
80 kDa
50 kDa
33 kDa
27 kDa
19 kDa
Adult Brain
50 kDa
33 kDa
27 kDa
Foetal Brain
R155
mBcl-2

E.


Markers
non IP
IP R155
IP R155 + peptide
IP pre-immune
IP R155
34 kDa
27 kDa
3F4
no first Ab
27 kDa
16.5 kDa
Bcl-2
no first Ab
FOETAL BRAIN

2. IN VIVO PrP^C AND Bcl-2 INTERACTION

2.1 Assessing the PrP^C-Bcl-2 interaction using the yeast-two-hybrid matchmaker system

To determine the PrP^C-Bcl-2 protein-protein interaction in vivo the yeast-two-hybrid matchmaker system was used. The eukaryotic yeast-two-hybrid matchmaker system is based on the properties of the GAL 4 protein of *Saccharomyces cerevisiae* yeast strains (Fields and Song, 1989). GAL 4 regulates the expression of genes encoding the enzymes required for galactose utilization as a carbon source. GAL 4 consists of two separable and functionally essential domains: an N-terminal domain which binds specific DNA regulatory sequences (UAS GAL 4) necessary for the transcription of the Lac Z reporter gene; and a C-terminal domain necessary for the transcriptional activation of the Lac Z gene (Guthrie et al, 1991; Guarente, 1993; Fields and Song, 1993). In the yeast-two-hybrid matchmaker system, the N-terminal and C-terminal domains of GAL-4 are encoded by the two vectors pGBT9 and pGAD424, respectively. pGBT9 is a 5.4 kb vector that encodes a DNA binding domain and pGAD424 (6.4 kb) encodes an activation domain. The PrP^C protein was cloned into the pGAD424 vector and the Bcl-2 protein was cloned the pGBT9 vector. Both the pGBT9-Bcl-2 and pGAD424-PrP constructs were co-transformed in the *Saccharomyces cerevisiae* yeast strain YRG 2 by electroporation. Other methods such as lithium acetate and polyethylene glycol were used to co-transform the yeast cells. However, electroporation yielded 10 times the number of co-transformants. Synthetic dropout (SD) media was used to isolate the co-transformants. SD media contains a yeast nitrogen base, a carbon source (2% glucose) and an amino acid dropout solution. Control YRG 2 cells that do not express any DNA, grew on SD agar plates containing all the essential nutrients and amino acids. Omission of histidine did not allow their growth, as expected. YRG 2 cells transformed with the pGBT9 plasmid grew on SD plates without tryptophan since the vector already expresses

the tryptophan marker (Trp 1). Omission of leucine from the SD medium selected for the pGAD424 control plasmid. Omission of tryptophan or leucine in growth media selected co-transformants of pGBT9 and pGAD424. Removal of leucine, tryptophan, and histidine selected plasmids encoding the interacting proteins. If the two proteins are interacting, transcription of the His 3 and Lac Z reporter genes of YRG 2 is induced.

β-galactosidase activity (table 3) was detected by qualitative colorimetric filter assays and quantitative β-galactosidase liquid culture assays (Bartel et al, 1993). According to the β-galactosidase colorimetric filter assay, PrP^C and Bcl-2 interact. A strong blue colour appeared on the filter indicating a positive interaction. Controls such as YRG 2 cells alone, the pGBT9 vector alone, and the pGAD 424 vector alone showed a white colour indicating that β-galactosidase activity was not activated by vector alone. pGAD424 x pGBT9, Bcl-2-pGBT9 x pGAD424, PrP-pGAD424 x pGBT9, PrP-pGAD424, and Bcl-2-pGBT9 controls showed a faint blue colour, indicating a possible false-activation of β-galactosidase. This is one limitation associated with the yeast-two-hybrid matchmaker system. To further confirm the PrP^C-Bcl-2 interaction, we used the more quantitative β-galactosidase liquid culture assays. In the presence of β-galactosidase activity, ONPG releases o-nitrophenol and D-galactose seen as a yellow colour and detected by spectrophotometry. β-galactosidase units were calculated using the following formula: β-gal units = 100x [OD 420/t x V x OD 600]. Table 3 shows the β-galactosidase units for each type of interaction. The average β-galactosidase units were calculated based on three individual clones assayed for each interaction. The PrP^C-Bcl-2 interaction represents a twenty-five-fold increase in β-galactosidase activity in comparison to the controls non-transformed yeast or yeast transformed with vectors on their own. Controls PrP^C-pGAD424 x pGBT9, Bcl-2-pGBT9 x pGAD424, Bcl-2-pGBT9, or PrP^C-

pGAD424 revealed a slight background β-galactosidase activity. However, PrP^C and Bcl-2 co-transformants displayed a three-fold increase in β-galactosidase activity in comparison to the controls.

Although the yeast-two hybrid matchmaker is a quick method for detecting protein-protein interactions, and has many advantages, there exist several limitations. This system is a highly sensitive and quick method for detecting *in vivo* protein-protein interactions that may not be detected by other means (Bartel et al., 1993). These interactions of proteins are detected within the native environment of the eukaryotic yeast cell and no biochemical purification of the proteins is required (Phizicky and Fields, 1995). The proteins in question must be able to fold and be stably expressed in the yeast cells (Phizicky and Fields, 1993). The proteins must be able to retain their activity as fusion proteins and since they are fused to vectors, their sites of interaction may be blocked (Phizicky and Fields, 1993). The β-galactosidase filter assay used to assess β-galactosidase activity gives many false-positive results since detection is based on colour formation (Phizicky and Fields, 1993). Quantitative β-galactosidase liquid clture assays may also leave room for inconsistencies since each culture differs in terms of the level of β-galactosidase activity, even if treated under the same conditions. In liquid phase, the yeast can also release their constructs.

Table 3.- Results from yeast-two-hybrid system

Table 3 summarizes results from the β-galactosidase colorimetric filter assay as well as the quantitative β-galactosidase liquid culture assay done using the yeast-two-hybrid matchmaker system. A strong blue colony (designated as +++) indicates a positive interaction. White colonies indicated an absence of interaction (designated as -). A faint blue coloured colony, is designated as a weak interaction (+/-). The liquid culture assays were done as described in materials and methods. Results are expressed as the mean β-galactosidase units ± standard deviation of three independant experiments. The β-galactosidase units of control no DNA were subtracted as background from all tested constructs.

Table 3. - YEAST MATCHMAKER 2-HYBRID SYSTEM RESULTS

INTERACTION	β-GAL UNITS	STD. DEV.	COLORIMETRIC FILTER ASSAY
NO DNA	0.0	0.0	–
pGBT9	0.0	3.6	–
pGAD424	0.0	4.9	–
pGBT9 x pGAD424	2.1	2.2	+/–
Bcl-2-pGBT9 x pGAD424 -	4.5	1.1	+/–
PrP-pGAD424 x pGBT9 -	0.9	1.4	+/–
PrP-pGAD424	9.7	2.4	–
Bcl-2-pGBT9	8.9	2.7	–
Bcl-2-pGBT9 x PrP-pGAD424	25.3	2.2	+++

β-gal units = 1000 × [OD 420/t × V × OD 600]
t= time of incubation (min.)
V= volume of culture (ml)

Legend: **-: no interaction (white colour)**
+/-: weak interaction (faint blue)
+++: strong interaction (blue)

2.2 Investigating the in vivo PrP^C-Bcl-2 interaction by crosslinking

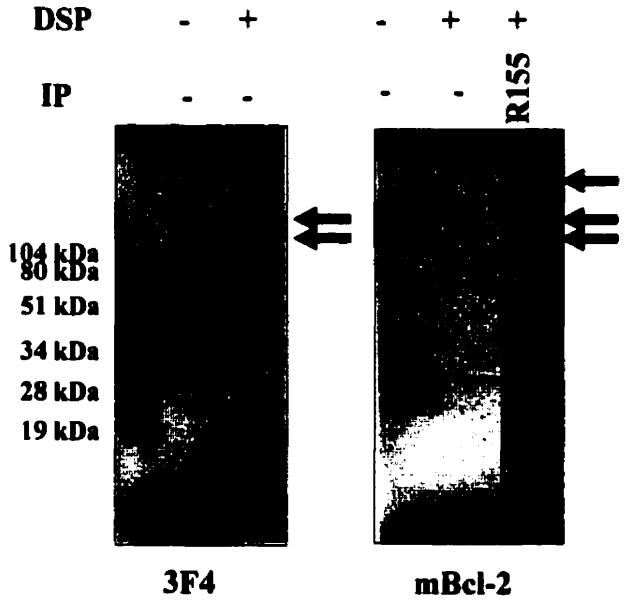
To further confirm the interaction of PrP^C and Bcl-2 in vivo, crosslinking experiments were performed. Crosslinking is a quick method for detecting protein-protein interactions between proteins that interact in a cell. In addition, crosslinking can detect weak interactions with different proteins. Crosslinking was accomplished using dithiobis-succimmidyl-proprionate (DSP), a membrane-permeable crosslinking reagent followed by immunoprecipitation of the ligand protein. DSP is an NHS-ester which interacts with the primary amine group of the protein ligand. Human primary foetal neurons known to express high levels of PrP^C and Bcl-2 were used for in vivo crosslinking experiments. The neurons were treated with DSP according to the manufacturer's specifications. Proteins were extracted, immunoprecipitations were carried out with anti-human PrP R155 antisera, separated on a 10% acrylamide gel under non-reducing conditions, and immunoblotted with monoclonal anti-human 3F4 and Bcl-2 antibodies (figure 10A). In the presence of DSP crosslinker, the amount of 35 kDa PrP^C is decreased. In addition, two faint bands above 104 kDa appear indicating that PrP^C has crosslinked to other proteins. The proteins bound to PrP^C may include Bcl-2 and other related proteins. To confirm the presence of Bcl-2, thirty times more protein was immunoprecipitated with polyclonal anti-human R155 (anti-PrP) antisera, and immunoblotted with monoclonal anti-human Bcl-2 antibody. The same two bands above 104 kDa were immunodetected indicating that Bcl-2 is crosslinked to PrP^C. In addition, several bands are detected at 34 kDa and above 104 kDa. These multiple bands may represent crosslinked proteins specifically immunoprecipitated with PrP^C and detected with Bcl-2. The band at 34 kDa may represent one isoform of PrP^C of a low molecular weight (less than 16 kDa as seen in figure 9A, in the primary human foetal neurons) associated with the 26 kDa Bcl-2 protein. The various sizes of the crosslinked proteins may indicate the association of various sized isoforms of PrP^C with Bcl-2. The reason why the

multiple bands above 104 kDa are faint may be because crosslinking can mask the epitopes recognized by the antibody or because there is aggregation of the proteins occurring, an event that usually accompanies crosslinking. To confirm that all of the protein was present in the detergent soluble fractions, detergent insoluble proteins from DSP-treated cultures were separated on 15% SDS PAGE non-reducing gels in parallel to untreated neurons. Results are shown in figure 10B. In the presence of DSP, the gradual disappearance of PrP^C and Bcl-2 is observed. However, no bands indicating crosslinking are observed. This result confirms the presence of all protein in the detergent soluble fractions. In conclusion, PrP^C and Bcl-2 interact in vivo.

Figure 10. Western blot analysis showing PrP^C-Bcl-2 interaction in primary human foetal neurons by crosslinking

A. Proteins extracted from primary human foetal neurons **(DSP -)** were separated on an SDS polyacrylamide gel under non-reducing conditions, along with proteins extracted from DSP-treated primary human foetal neurons **(DSP +)**. Monoclonal anti-human PrP **(3F4)** and Bcl-2 **(mBcl-2)** antibodies were used for immunoblotting. The last lane of the right panel of figure 10A represents an immunprecipitation of thirty times more protein with R155 **(IP + , R155)**. The same two bands (above 104 kDa) that were present in the non-immunoprecipitated samples **(IP -)**, are seen at a much higher intensity. **B.** The proteins extracted from the detergent-insoluble fractions of both DSP-treated **(+)** or untreated **(-)** neurons were separated on an SDS polyacrylamide gel. PrP and Bcl-2 were detected using monoclonal anti-human PrP **(3F4)** and Bcl-2 **(mBcl-2)** antibodies.

A.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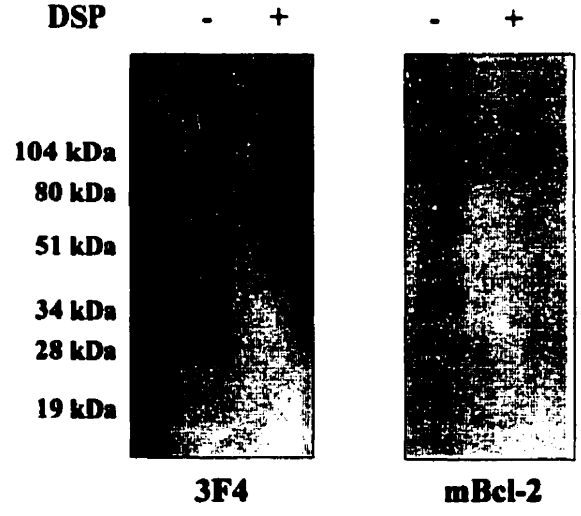
80 kDa
51 kDa
34 kDa
28 kDa
19 kDa
3F4
mBcl-2

B.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104 kDa
80 kDa
51 kDa
34 kDa
28 kDa
19 kDa
3F4
mBcl-2

3. Interaction of PrP^C and Bax in human brain

The 21 kDa pro-apoptotic protein Bax is a heterodimerizing partner of Bcl-2. Bax shares 21% homology with Bcl-2 and this homology lies in the BH1 and BH2 domains. Since Bax is also highly expressed in the central nervous system, and the amino acid sequence of the BH2 domain of Bax is highly homologous to the amino acid sequence of the octapeptide repeats of the PrP^C molecule , the interaction between Bax and PrP^C was also investigated. The PrP^C-Bax interaction was studied both in a cell-free system as well as within living cells by methods of co-immunoprecipitation and crosslinking. Figure 11A represents results from the co-immunoprecipitation of Bax and PrP^C in human foetal and adult brain. Proteins (150 μg) extracted from whole foetal brain tissue were immunoprecipitated with polyclonal anti-human Bax antisera and immunoblotted with monoclonal anti-human 3F4 antibody. All isoforms of PrP^C (25-34 kDa) as present in non-immunoprecipitated controls (- lane) are detected. The same result was obtained with the proteins from the adult human brain. As expected, less PrP^C co-immunoprecipitates with Bax in the adult human brain since Bax levels are low in the adult brain and less Bax is available to be bound by PrP^C as shown later (figure 13). Figure 11B shows the western blot analysis of proteins extracted from adult brain immunoprecipitated with polyclonal anti-human R155 antisera and immunoblotted with polyclonal anti-human Bax antibody. A 21 kDa band representing Bax , as present in the non-immunoprecipitated control, (-) is detected. A thick smear-like pattern is also seen. This smear is probably due to the problems of IgG detection by the secondary antibodies when using polyclonal antibodies. To confirm this possibility, we bought a monoclonal Bax antibody and repeated the experiments (Figure 11C). To confirm that the bands detected by the Bax antisera were specific, primary antibody control experiments were performed, and monoclonal anti-human Bax and anti-human 3F4 antibodies were used for

immunoblotting. Results are shown in figure 11C. Immunoprecipitated Bax is detected as one band with the monoclonal Bax. The co-immunoprecipitation of PrP^C is confirmed with 3F4 immunostaining. Addition of a specific bax peptide against the Bax antibody eliminates the immunodetection of both Bax and PrP^C indicating that the Bax peptide successfully competes out the immunoprecipitation.

The PrP^C-Bax interaction was confirmed in vivo using DSP crosslinking agent on human primary foetal neurons. In the presence DSP, there is a gradual disappearance of the Bax protein, indicating that the Bax protein has crosslinked (refer to figure 12). When thirty times more protein were immunoprecipitated with monoclonal 3F4 antisera, bands at 51 kDa, 34 kDa, and slightly above 21 kDa are detected. The band at 51 kDa may represent Bax-PrP^C bound proteins since the size of the band corresponds to the additive molecular weights of both proteins. The faint band present slightly above 21 kDa could be Bax. The reason for the band shifting in the acrylamide gel is probably due to the fact that it may be bound to some small post-translationally modified isoform of PrP^C below 16 kDa. The same explanation could be given for the sharp band present at 34 kDa.

On the basis of both the in vivo and in vitro co-immunoprecipitation and crosslinking experiments PrP^C and Bax interact in human neurons.

Figure 11. Western blot analysis showing interaction of Bax and PrP^C in human foetal and adult brain

A. Western blot analysis showing co-immunoprecipitation of Bax and PrP^C in human foetal and adult brain

Proteins (150 μg) extracted from either foetal or adult human brain were immunoprecipitated with polyclonal anti-human Bax antisera **(Bax).** Immunoprecipitates were separated on an SDS polyacrylamide gel in parallel to 40 μg non-immunoprecipitated samples **(IP -)**. Westerns were probed with 3F4.

B. Western blot analysis showing co-immunoprecipitation of Bax and PrP^C in human adult brain

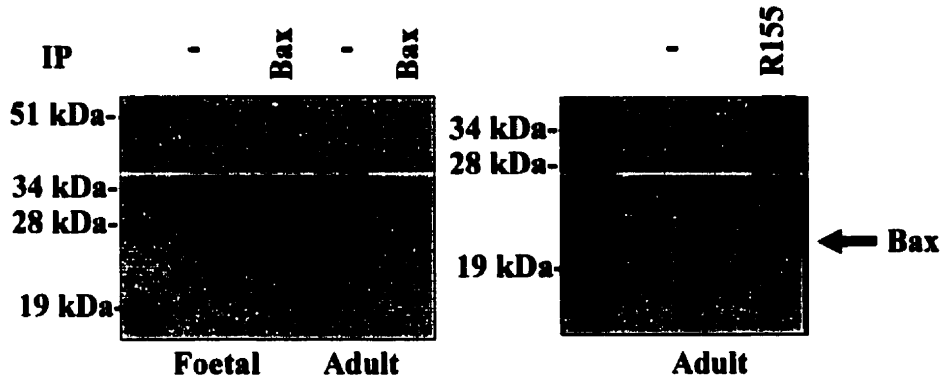
Proteins (150 μg) extracted from adult human brain were immunoprecipitated with polyclonal anti-human PrP antisera **(R155)**. As was done in A, immunoprecipitates were separated on an SDS polyacrylamide gel in parallel to 40 μg non-immunoprecipitated samples **(IP -)**. Westerns were probed with polyclonal Bax **(pBax)**.

C. Controls done for the co-immunoprecipitation of Bax-PrP^C in human foetal brain

In the upper panel, the first lane shows 40 μg proteins extracted from human foetal brain non-immunoprecipitated **(non-IP)**. The other lanes show 150 μg proteins immunoprecipitated with polyclonal Bax antisera **(IP pBax)**, polyclonal Bax antisera competed with a peptide against Bax **(IP pBax + peptide)** or pre-immune antisera **(IP pre-immune)**. All were immunoblotted with monoclonal 3F4 antibody. Lane 5 shows 150 μg proteins immunoprecipitated with polyclonal Bax antisera and no 3F4 antibody used for probing **(no first Ab)**. The same experiment was done using monoclonal Bax for immunoblotting (lower panel).

A.

B.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Bax
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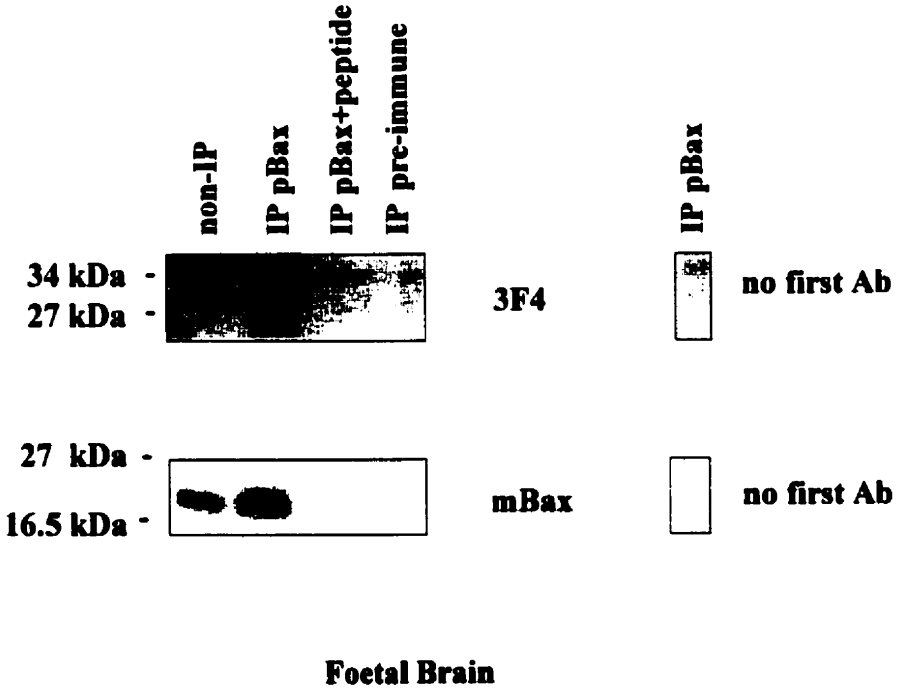
pBax

C.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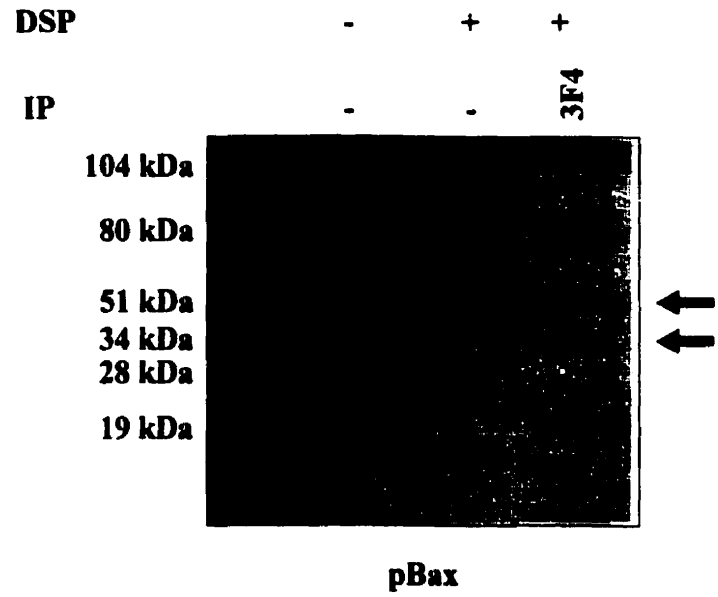
IP pBax+peptide
IP pre-immune
34 kDa -
27 kDa -
3F4
IP pBax
no first Ab
27 kDa -
16.5 kDa -
mBax
no first Ab
Foetal Brain

Figure 12.Western blot analysis showing the in vivo interaction of PrP^C and Bax in primary human foetal neurons using crosslinking

Proteins extracted from primary human foetal neurons **(DSP -)** were separated on an SDS polyacrylamide gel under non-reducing conditions, along with proteins extracted from DSP-treated primary human neurons **(DSP +)**. Polyclonal anti-human Bax antibody **(pBax)** was used for immunoblotting. The last lane of the western blot represents an immunoprecipitation of thirty times more protein with the monoclonal anti-human PrP antisera **(IP 3F4)**.


DSP
-
+
+
IP
-
-
3F4
104 kDa
80 kDa
51 kDa
34 kDa
28 kDa
19 kDa
pBax

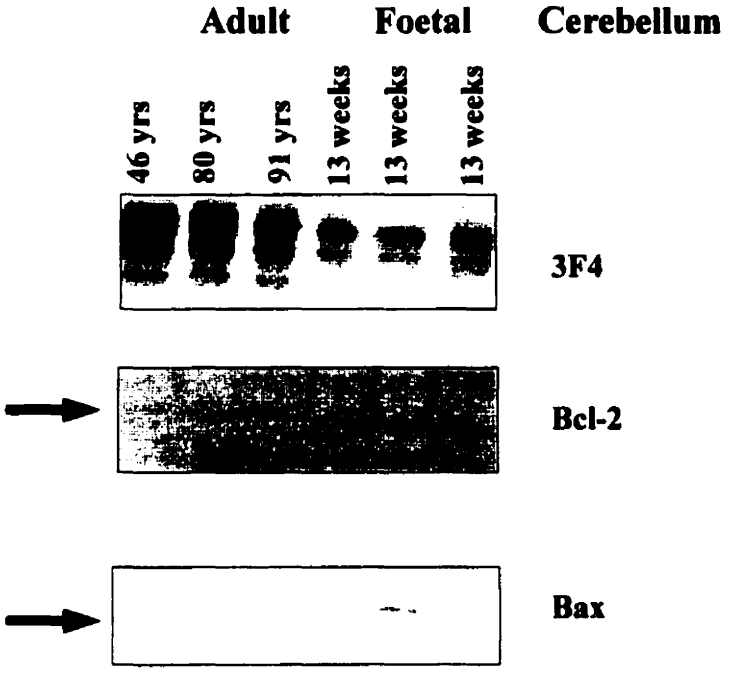
4. Expresssion patterns of PrP^C, Bcl-2, Bax in human brain

The levels of expression for PrP^C, Bcl-2, and Bax were studied in the cerebellum of human foetal brains aged 13 weeks old as well as in human adult brains of 46 year old, 80 year old, and 91 year old individuals. The reason for chosing the cerebellum for our study was to compare the expression patterns of PrP^C, Bcl-2, and Bax during development within a specific region of human brain. Also, high levels of PrP^C and Bcl-2 are expressed in the Purkinge neurons of the cerebellum. Proteins extracted (40 μg) from the post-mortem tissues were separated by 15% SDS-PAGE, and immunoblotted with monoclonal anti-human 3F4, Bcl-2, and polyclonal anti-human Bax antibodies respectively. According to figure 13, PrP^C expression increases with age. A more abundant expression of the PrP^C protein (represented by bands between 16 kDa-35 kDa) is noted in the adult human brain cerebellum in comparison to the foetal cerebellum. Results are consistent with those previously seen (figure 9A). Additional isoforms of the PrP^C protein (sharp bands below 16 kDa), which may correspond to the proteolytically cleaved N-terminal fragments of PrP^C exist in the adult stages. The higher expression of PrP^C in the aged CNS is expected since prion diseases normally manifest themselves past the third or fourth decade of life. Figure 13 also depicts the developmental expression of Bcl-2. In mice, Bcl-2 expression decreases with age (Merry et al., 1994 ; reviewed by Reed, 1994). Similar to Bcl-2, Bax expression also decreases with age (refer to bottom western blot of figure 13). Therefore, we conclude that PrP^C and Bcl-2 interact in human foetal and adult brain, and foetal neurons. The interaction of PrP^C, Bax, and Bcl-2 and the expression patterns of PrP^C, Bax, and Bcl-2 indicate that PrP^C may be a member of the Bcl-2 family of proteins. An inverse relationship with respect to the expression of Bcl-2, Bax, and PrP^C occurs with age in the aging CNS. PrP^C may functionally replace Bcl-2 or Bax in the aging CNS. Different modifications of PrP^C such as

conformational changes, or mutations may alter the interactions of PrP^C with Bcl-2 and Bax. The increased expression of PrP^C during aging could explain why prion diseases involving mutations do not manifest themselves before the third or fourth decade of life.

Figure13. Different expression patterns of PrP^C, Bcl-2, and Bax in the CNS

Each lane of this western blot represents 40 μg of proteins extracted from both human foetal (aged 13 weeks) and human adult cerebellum (46, 80, 91 years of age). Monoclonal anti-human PrP (3F4), Bcl-2, and Bax antibodies were used for immunoblotting. An inverse relationship exists between the expression patterns of PrP^C, Bcl-2, and Bax. In contrast to PrP^C expression, Bcl-2 and Bax expression decreases with age in the CNS.


Adult
Foetal
Cerebellum
46 yrs
80 yrs
91 yrs
13 weeks
13 weeks
13 weeks
3F4
Bcl-2
Bax

IV. Discussion

1. Apoptosis and prion diseases

Programmed cell death (apoptosis) is a normal physiological process which maintains homeostasis in neurons during the development of the human central nervous system (CNS). Any dysregulation in the programmed cell death cycle may result in the death of long-lived, terminally differentiated neurons. Neurodegenerative disorders including prion diseases may partly arise from dysregulated apoptosis. Prominent hallmarks of apoptosis such as DNA fragmentation, and cell shrinking, have been observed in scrapie-infected sheep brain as well as post-mortem brains of humans afflicted with CJD, GSS, FFI, FASE, and variants of human prion disorders. The neurodegeneration, dementia, and spongiform encephalopathy associated with prion diseases have been attributed to the overexpression of PrP^C, the altered biogenesis and metabolism of PrP^C, and mutations in PrP^C, which result in its conformational conversion to the infectious and transmissible scrapie isoform (PrP^{SC}) (reviewed by Collinge, 1997). However, the normal cellular function of PrP^C and the mechanism of PrP^{SC} remains unclear. Studies conducted in transgenic and PrP^C knockout mice implicate PrP^C in astrocyte hypertrophy and proliferation, normal synaptic transmission, as well as neuronal differentiation and CNS maturation (Cashman et al., 1990; Manson et al., 1992; Collinge et al., 1994). Apoptosis, being a critical component of neuronal development and CNS maturation, may be associated with the PrP^C protein. This notion raises several important questions. Does PrP^C modulate the fate of neurons? Is PrP^C a regulator of apoptosis and how? The role of PrP^C in apoptosis has never been addressed. In the present study, we show that PrP^C interacts with Bcl-2 and Bax, two key modulators of apoptosis. Identifying proteins that interact with PrP^C is a good starting point to assign a precise function to PrP^C and understand PrP^C's implications in neurodegenerative diseases.

1.1 Prion protein interacts with Bcl-2

Dr. Andrea LeBlanc noticed that the amino acid sequence of the octapeptide repeats in the N-terminus of PrP^C, is homologous to the amino acid sequence of the BH2 domain of the anti-apoptotic Bcl-2 protein. The homology existing between the PrP^C and Bcl-2 proteins indicates a possible membership of $PrP^C$to the Bcl-2 family of proteins. Bcl-2 and related proteins are implicated in the regulation of apoptosis. In the central nervous system, Bcl-2 protects neurons from apoptotic stimuli such as deprivation of NGF, toxic Ca^{2+} levels, and oxidative stress (Allsopp et al., 1993; Zhong et al., 1993; reviewed by Wyllie et al., 1993; reviewed by Reed, 1994; Martinou et al., 1994; Farlie et al., 1995; Yang and Korsemeyer, 1996). The exact mechanism of action of Bcl-2 is unclear. However, it is known that the BH2 domain of Bcl-2 confers its anti-apoptotic functions and allows it to interact with an array of proteins. In particular, Bcl-2 interacts with the cell death protein Bax. In a cell, Bcl-2 heterodimerizes with Bax and rescues cells from apoptosis. The ratio of Bcl-2-Bax heterodimers versus Bax-Bax homodimers determines the susceptibility of a cell to apoptotic stimuli such as those mentioned above (Yin et al., 1994; Oltvai and Korsemeyer, 1994). Since neuronal cell death lies at the heart of neurodegenerative disorders such as prion diseases of both humans and animals, it is possible that PrP^C is implicated in regulating apoptosis through Bcl-2 and related proteins.

In the present study we show the PrP^C-Bcl-2 interaction by co-immunoprecipitation both in PrP^C-transfected human erythrolymphoid K562 cells as well as in post-mortem human foetal and adult brains. The <u>in vivo</u> interaction of $PrP^C$and Bcl-2 is shown by the yeast-two-hybrid matchmaker system and crosslinking in human primary foetal neurons. The yeast-two-hybrid matchmaker system, although a quick method to detect protein-protein interactions, leaves room for inconsistencies. False activation of β-galactosidase may result even in the presence of appropriate controls. Crosslinking, on the other hand, is a much better

method for detecting protein-protein interactions in vivo. However, masking of the epitopes recognized by antibodies usually accompanies crosslinking. In our case, the crosslinked proteins were evident as faint bands due to masking of the epitopes recognized by anti-human PrP^C and Bcl-2 antibodies.

Despite the technical difficulties encountered with both the yeast-two-hybrid matchmaker system and crosslinking, our data clearly indicate an interaction of $PrP^C$and Bcl-2 both in vitro and in vivo. The cell systems we used to investigate the PrP^C-Bcl-2 interaction are appropriate since both proteins are highly expressed in the human CNS and prion diseases are restricted to the CNS. Our studies provide novel information about both PrP^C and Bcl-2 since the interaction of PrP^C and Bcl-2 has never been investigated in human neurons.

In order to understand the significance of the interaction between the PrP^C and Bcl-2 proteins, it is necessary to know where the interaction takes place both within the cell as well as at the molecular level. One prerequisite for the interaction of PrP^C with Bcl-2 is that the two proteins must co-exist in the same cell. It is clearly evident from our data that PrP^C and Bcl-2 are highly expressed in neurons of the human CNS making it possible for the proteins to associate with each other. However, the subcellular compartment in which PrP^C and Bcl-2 interact is unknown. Pinpointing the exact subcellular location of the PrP^C-Bcl-2 interaction is a future goal. Methods such as subcellular fractionation and immunocytochemistry have already been used to address this issue. Preliminary results from a subcellular fractionation experiment done in human primary foetal neurons reveals that $PrP^C$and Bcl-2 are co-localized with calnexin, an endoplasmic reticulum (ER)-specific chaperone, in the ER fraction (unpublished data and not shown in present thesis, Papadopoulos, M and LeBlanc, A.C.). In support of such findings, is immunocytochemistry done using specific anti-human monoclonal and polyclonal Bcl-2 and PrP^C antibodies indicating co-localization of PrP^C and Bcl-2

in a perinuclear region consistent with the ER location (unpublished data and not shown in present thesis, LeBlanc, A.C.). Further experiments of both immunocytochemistry and subcellular fractionation must be done to confirm these findings and to draw clear conclusions.

The recent evidence that PrP^C can be synthesized as a transmembrane form at the ER membrane lends support to our findings. C-transmembrane PrP^C has its C-terminus directed in the ER lumen while the bulk of the protein is exposed to the cytosol of cells (Hegde et al., 1998). Therefore, this topography and localization of PrP^C in the ER would allow interaction with Bcl-2. Bcl-2 is also localized in the ER (Zhu et al., 1996).

Dysregulation in the biogenesis of PrP^C, or mutations in specific stop transfer effector sequences (STE) or transmembrane regions of the PrP^C gene which are responsable for regulating the translocation of PrP^C at the ER membrane, result in overexpression of C-transmembrane PrP^C. C-transmembrane PrP^C protein subsequently accumulates in the CNS, and causes neurodegeneration (DeFea et al., 1994; Hegde et al., 1998). We propose a model of PrP^C interacting with Bcl-2 as depicted in figure 14.

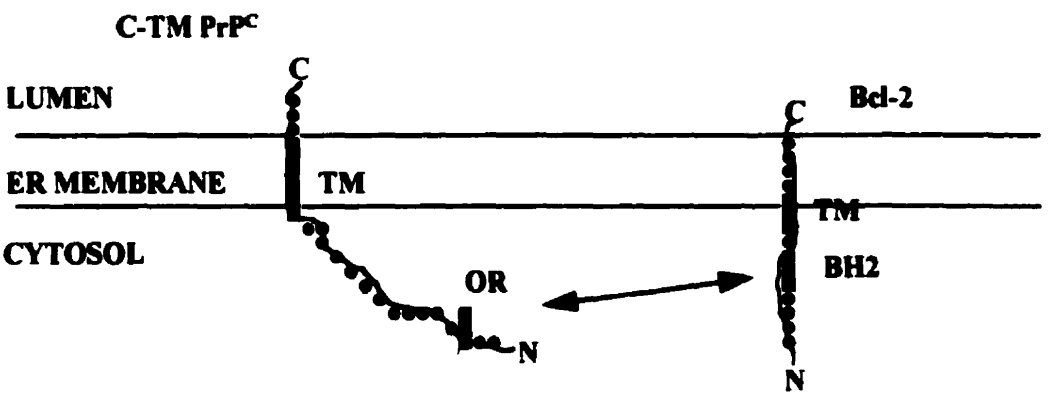


Figure 14.-Model of PrP^C-Bcl-2 interactions

According to figure 14, C-transmembrane PrP^C(C-TM PrP^C) binds to Bcl-2 when Bcl-2 is localized to the ER. As discussed in the introduction of the present thesis, Bcl-2's subcellular location is cell-type dependant. Bcl-2 can exist either as an integral membrane protein or cytosolic protein. In our model, Bcl-2 is attached to the ER membrane via its carboxyl terminus while the bulk of the protein is exposed to the cytosol. Through its BH2 domain, Bcl-2 binds to the N-terminus of PrP^C where the octapeptide repeats (OR) are localized.

Since there is homology between the BH2 domain of Bcl-2 and the octapeptide repeats of PrP^C, and the BH2 domain allows Bcl-2 to interact with different proteins, we expect the interaction to be taking place between the respective sites. However, the present study does not specifically show this. Site-directed mutagenesis of the BH2 domain of Bcl-2 or even the octapeptide repeats of PrP^C is one experiment to be done in the future. Once mutants are created, it is necessary to test the different interactions by co-immunoprecipitation and crosslinking in vivo and in vitro. In addition, truncated constructs of PrP^C and Bcl-2 can be used to test the interactions between the two proteins and assess the exact site of interaction between the molecules. In 1995, Kurshner and Morgan screened a cerebellar cDNA library with the goal of identifying specific neuronal Bcl-2-binding proteins. Full length murine Bcl-2 was used as bait. Kurshner and Morgan reported an interaction between amino acids 72-254 (includes the octapeptide repeats) of the murine PrP^C and amino acids 176-236 (including the BH2 domain) of the carboxyl terminus of murine Bcl-2 in the yeast-two-hybrid system. The results of Kurshner and Morgan support our idea that interaction occurs between the BH2 domain of Bcl-2 and the octapeptide repeats of PrP^C. In contrast to their studies, we have used full-length human PrP^C and Bcl-2 proteins thereby avoiding possible binding artifacts due to the conformation of peptides. Also, we have studied the PrP^C-Bcl-2 interaction in vitro in K562 cells and in human brain and in

vivo in human primary foetal neurons demonstrating physiological significance to these findings.

What is the significance of the PrP^C-Bcl-2 interactions? Several theories can be postulated regarding the significance of the PrP^C-Bcl-2 interaction and the effects of this interaction on neuronal cells.

First, it is possible that PrP^C interacts with Bcl-2 and helps target Bcl-2 to the endoplasmic reticulum membrane. At the ER, the anti-apoptotic activities of Bcl-2 are enhanced. Zhu et al specifically targetted Bcl-2 to different subcellular locations and they found that once in the ER, Bcl-2 was able to bind Bax more easily and its anti-apoptotic functions were enhanced (Zhu et al., 1996). Although Zhu et al conducted their studies in MDCK cells, the same may occur in human neurons which are also polarized cell types. Scientists have not investigated this issue yet. PrP^C, allowing Bcl-2 to carry out its anti-apoptotic functions, could be beneficial to neurons. Any mutations to PrP^C or alterations in its synthesis, metabolism, or conformation, may abrogate PrP^C's role in neuroprotection and neurodegeneration may result.

Second, the PrP^C interaction with Bcl-2 may displace Bax and disrupt the formation of Bcl-2-Bax heterodimers. As was previously discussed, Bcl-2-Bax heterodimers protect neurons from apoptosis. If the formation of Bcl-2-Bax heterodimers is abrogated by the PrP^C protein which binds Bcl-2, Bax is left free in the cell. Overexpression of Bax as Bax monomers or Bax-Bax homodimers promotes neuronal cell death as seen in patients with prion disease. Overexpression of PrP^C and perhaps mutant PrP, may also offset the balance between the death and survival in neurons by inhibiting Bcl-2-Bax heterodimers.

Third, Bcl-2 may be a bridging protein involved in the PrP^{C}-PrP^{SC} conversion.The exact mechanism by which prion infectivity increases is unknown. The "protein only" hypothesis is most accepted in explaining the processes involved in the development and transmissibility of prion diseases. According to the "protein only" hypothesis, PrP^{C} is converted into PrP^{SC} in an exponential process. PrP^{SC} subsequently accumulates in the CNS and results in diseases (Prusiner, 1991; reviewed by Prusiner, 1996). Prusiner and colleagues have recently reported that a yet uncharacterized protein (protein X) is a probable intermediate involved in the PrP^{C} to PrP^{SC} conversion (Prusiner, 1991). Bcl-2 may be protein X (Kurshner and Morgan, 1995;1996) . This idea was addressed in the paper by Kurshner and Morgan. It has also been reported that foldases and molecular chaperones may also facilitate the PrP^{C} to PrP^{SC} conversion (Prusiner, 1991).

In summary, there are several implications of the interactions of PrP^{C} with Bcl-2 are several. The negative cellular effects of such interactions outnumber the positive effects. Disruption of protein-protein interactions between PrP^{C}-Bcl-2 by either mutations, overexpression, or conformational changes may result in abrogation or alteration in each of the functions of the proteins concerned. As a result, neurodegeneration may occur.

1.2 PrP^{C} interacts with Bax

Since Bax is a heterodimerizing partner of Bcl-2 and its BH2 domain is homologous to the octapeptide repeats of PrP^{C}, we expected Bax to interact with PrP^{C}. However, Kurshner and Morgan (1996) reported no interaction between Bax and PrP^{C}. Their conclusion was based on one negative colorimetric β-galactosidase filter assay using the yeast-two-hybrid matchmaker system and the absence of co-precipitation in murine neuronal cultures. Such findings may be valid in murine neurons but the situation is not entirely reflective of human neurons. In

contrast, we clearly detect interaction of PrP^C with Bax by co-immunoprecipitation in human foetal and adult brain as well as crosslinking in human primary foetal neurons. It cannot be emphasized enough that we studied the interaction of full-length human PrP^C and Bax proteins in human foetal and adult brain in addition to human neurons in vivo and in vitro. In contrast to Kurshner and Morgan, we conclude that PrP^C and Bax do interact.

How can PrP^C interact with both Bcl-2 and Bax? A possible explanation for PrP^C's involvement with both Bcl-2 and Bax is that PrP^C has four octapeptide repeats and one nonapeptide repeat of related sequence. The amino acid sequence of the BH2 domain of both Bcl-2 and Bax proteins is homologous to all the repeats of PrP^C. PrP^C may bind to the BH2 domain of Bcl-2 via one octapeptide repeat while binding to Bax through a second octapeptide repeat. Since these are all small proteins of 21-26 kDa, steric hindrance may not present a problem.

Again, a prerequisite for a significant interaction of PrP^C with Bcl-2 and Bax is that the proteins must co-exist in the same cell. Preliminary results from a subcellular fractionation experiment done in human primary foetal neurons reveal a co-localization of all three proteins in the ER (data not shown in present thesis). We have yet to confirm the co-localization by immunocytochemistrty.We propose the following model for the interaction of PrP^C with both Bcl-2 and Bax:

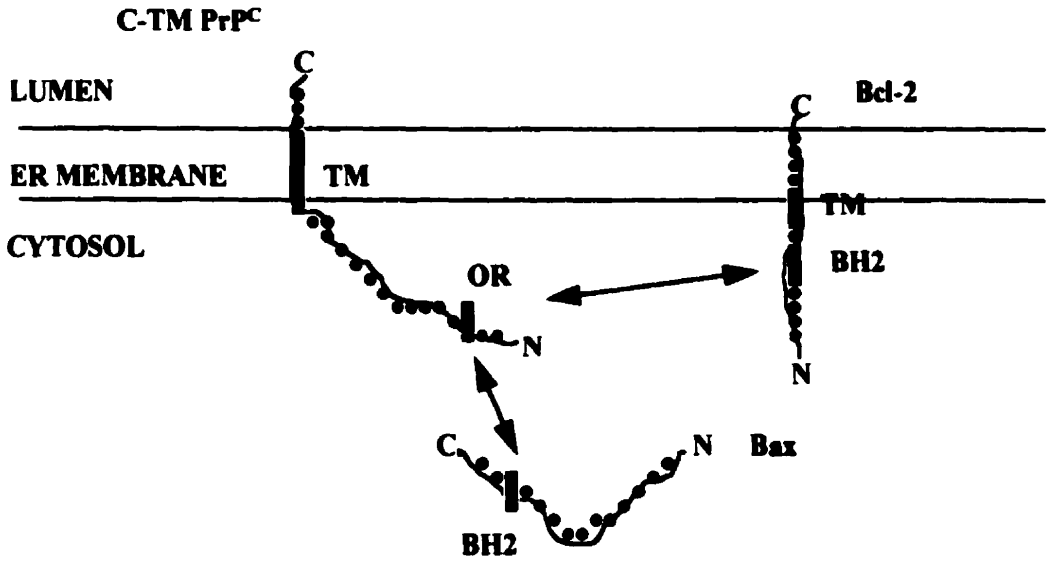


Figure 15.- Protein-protein interactions between PrP^C, Bcl-2, and Bax

According to figure 15, C-transmembrane PrP^C has its N-terminus in the cytosol and can bind to Bax since Bax is cytosolic. At the same time, PrP^C can also bind Bcl-2. As mentioned previously, Bcl-2 can exist either as an integral membrane protein or cytosolic protein. In both forms, Bcl-2 can interact with Bax (Zhu et al., 1996). PrP^C may be an intermediate between the Bcl-2-Bax interactions and may modulate the interactions taking place between Bcl-2 and Bax. PrP^C may either promote interactions between Bcl-2 and Bax by bringing the two proteins closer together or abrogate their interactions by preventing them from coming together. Through its interactions with Bcl-2 and Bax, PrP^C may modulate the death and survival states of neurons. Any disruption of such interactions by either overexpression, mutations, inoculation of PrP^{SC}, or altered expressions may lead to neurodegeneration as seen in prion diseases.

Future goals include testing the interaction of mutant PrP with Bcl-2 and Bax to determine the exact site of interaction within each protein and to determine the effect of PrP mutations associated with human Prion diseases on this interaction. In addition, we must confirm the function of these interactions.

Collectively our findings and propositions as outlined above, can be considered as a first step into assigning a role for PrP^C in apoptosis. This issue has never been addressed before. We clearly show that PrP^C interacts with key regulators of apoptosis Bcl-2 and Bax. The model that we have proposed explains how the proteins may interact and where this interaction may be taking place. Although the exact outcome of such interactions is not known yet, our ideas can be tested in the future by further experiments to draw final conclusions.

2.0 Developmental expression of PrP^C, Bcl-2, and Bax possibly affects the modulation of neuronal survival and prion disease manifestation

Once it was determined that PrP^C interacts with Bcl-2 and Bax proteins in human brain by crosslinking and co-immunoprecipitation in vivo and in vitro, the relationship between their expression levels in the human CNS was assessed. There is a more abundant expression of PrP^C in the human adult cerebellum in comparison to human foetal cerebellum. The increase in PrP^C expression with the development of the human CNS is consistent with previous studies undertaken by Manson et al. Northern blot analysis and in situ hybridization studies of PrP^C expression in mouse and hamster brains have revealed that PrP mRNA expression parallels the development of the CNS. Low levels of PrP^C were detected in both mouse and hamster brains respectively after the immediate post-natal period but increased four-fold to a high level maintained in the adult CNS. Constitutive expression of PrP^C in the adult brain indicates an important role for PrP^C in neural function and differentiation, as well as brain maturation (Manson et al., 1994).

Since our studies were conducted in human brain, they provide novel information about the expression of PrP^C in the human CNS.

In contrast to PrP^C, expression of the Bcl-2 and Bax proteins decreases with aging in the human CNS. In support of our results are Northern blot analysis and in situ hybridization assays indicating that Bcl-2 expression in the CNS is developmentally regulated. Strong Bcl-2 immunoreactivity is present in the embryonic stages of rat brain and during the first post-natal week but decreases with age. Very low levels of Bcl-2, usually undetectable by immunohistochemistry is seen in the adult brain (Merry et al., 1994). Our studies show the same pattern of Bcl-2 expression in the human CNS. In addition, Bax expression levels also decrease with age in the CNS.

What is the significance of the inverse relationship of expression between PrP^C, Bcl-2, and Bax in the human CNS? Could this explain the molecular processes underlying the manifestation and transmissibilty of prion disease?.There are several theories that can be postulated based on the variable expression of PrP^C, Bcl-2, and Bax in the developing CNS.

First, the inverse relationship between PrP^C, Bcl-2, and Bax expression levels implies that normal PrP^C may compensate for the loss of Bcl-2 anti-apoptotic functions in the adult CNS. On the other hand, PrP^C may acquire the pro-apoptotic functions of Bax. To determine the precise functional role of PrP^C and if PrP^C is a functional homologue of either Bcl-2 or Bax , an assay demonstrating the role of PrP^C in apoptosis must be done.

A decrease in the expression of Bcl-2 and an increase in PrP^C expression in the adult CNS may account for the restriction of prion diseases to the CNS. PrP^C, a ubiquitously expressed protein is highly expressed in the CNS. Significant levels of PrP^C have also been detected in heart, lung, and liver. Bcl-2 and Bax are also ubiquitously expressed proteins. However, the expression of PrP^C , Bcl-2, and

Bax is developmentally regulated only in the CNS, explaining the restriction of prion diseases in the CNS. In addition, the developmental expression of PrP^C, Bcl-2, and Bax in the CNS, may account for the manifestation of prion diseases in the third or fourth decade of life. As individuals age, Bcl-2 levels in the CNS decline, no longer providing neuroprotection. In contrast to Bcl-2, the expression of PrP^C, the central protein in prion disease pathogenesis, increases. Altered expression of PrP^C such as overexpression, may have an impact on the expression of both Bcl-2 and Bax. Different amounts of PrP^C, Bcl-2, and Bax may have an effect on the ratio between PrP^C-Bcl-2 and PrP^C-Bax dimers. An imbalance in the ratios may have an effect on the interactions between all three proteins. If interactions of PrP^C with Bcl-2 or Bax are involved in modulating neuronal death and survival, alterations in these may explain the neurodegeneration underlying prion diseases.

Transmission of prion diseases between different mammalian species is limited by a species barrier (reviewed by Prusiner, 1996; Collinge, 1996). The species-barrier in transmissibility of prion diseases may be explained by the existence of species-specific sequences of PrP^C, Bcl-2, and Bax. The variability of phenotypes associated with the different prion disorders can be explained by the variable expression of PrP^C, Bcl-2, and Bax proteins in the brain and during CNS development.

V. Conclusions

In conclusion, we have studied the protein-protein interactions of PrP^C with Bcl-2 and Bax in human foetal brain, human aduit brain, and primary human foetal neurons.Collectively, our results indicate that PrP^C interacts with both Bcl-2 and Bax proteins. In addition, PrP^C, Bcl-2, and Bax proteins are expressed in neurons of the foetal, developing, and adult CNS, making it possible for the proteins to associate with each other. Through its interactions with Bcl-2 and Bax, PrP^C may be implicated in the regulation of apoptosis or cell survival as a member of the Bcl-2 family of proteins.

Prion protein gene mutations, post-translational modifications of PrP^C, overexpression of PrP^C, altered expression levels of PrP^C, Bcl-2, and Bax, or inoculation with PrP^{SC}, may disrupt normal interactions between PrP^C, Bcl-2, and Bax proteins. If protein-protein interactions between PrP^C, Bcl-2, and Bax proteins are involved in the regulation of neuronal survival and death, disruption of these may result in neurodegenerative disease.

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