PrP catabolites as determinants of TSE susceptibility

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Contents

Declaration

I declare that this thesis has been completed entirely by myself and that the work presented herein is my own. All experiments were designed and carried out by myself, in collaboration with my supervisors. No part of this thesis has, or will be, submitted for any other degree, diploma or qualification.

Charmaine Love

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Abstract

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that are characterised by long incubation periods, protein aggregation and vacuolation. During TSE pathogenesis the normal, cellular prion protein, (PrP^C), which is encoded by the gene *PRNP,* misfolds and accumulates as abnormal disease associated prion protein, (PrP^{Sc}) within the central nervous system. Variants of the Prion protein gene are associated with susceptibility to TSE disease. For example sheep scrapie disease is modulated by several *PRNP* alleles, with certain alleles carried by susceptible animals being different from those carried by resistant animals. The mechanisms linking *PRNP* genetics and disease is poorly understood but may involve protein sequence, PrP^C expression levels, and possibly differences in protein processing.

Post-translational modification of PrP^C leads to specific cleavage (alpha cleavage) between amino acids 115/116 of ovine PrP, producing two fragments C1 and N1. Cleavage of PrP may occur as a protective mechanism, as a response to changes in the cellular environment or as a feature of an as yet unknown biological function. In the context of TSEs, alpha cleavage may inadvertently provide a protective role by reducing available PrP^C protein for conversion into PrP^{Sc}, assuming that the C1 fragment would be an inefficient substrate for conversion, the opposite theory was also proposed.

The former hypothesis became the focus of this present study, with the idea that total full-length PrP^C, total C1 or the ratio between full-length PrP^C and C1 may be linked to differences in scrapie susceptibility. To investigate these aims the C1 fragment was measured as a percentage of total PrP^C in different *PRNP* genotypes with varying degrees of susceptibility to scrapie and in different brain regions. This study found that PrP^C alpha cleavage increased during development from the new born lamb to the adult sheep, which may have consequences for the susceptibility differences related to age. There are also variations in the amount of alpha cleavage

between brain regions such as cortex and medulla that may influence scrapie strain targeting. Overall the amount of the C1 fragment in the different brain areas varied as much as 10x (range 5% to 60%). There was a significant difference in the ratio of C1 to the other PrP^C forms between two *PRNP* genotype groups carrying the VRQ and ARQ allele but there was no correlation between C1 level and scrapie susceptibility or scrapie incubation period in our scrapie models.

Alpha cleavage of PrP^C also occurs in various transgenic mouse models expressing different ruminant PrP sequences. In PrP^C over-expressing transgenic mouse models a higher ratio of C1 was observed, this may suggest a link between PrP^C expression levels and alpha cleavage. Transgenic mice are therefore important models to further investigate the link between PrP^C biology and scrapie disease phenotype.

In conclusion, this thesis has shown for the first time that certain ovine *PRNP* alleles can influence alpha cleavage of the PrP^C protein; however it appears not to be a significant indicator of TSE disease susceptibility in sheep.

Abbreviations

ADAMs	A disintegrin and metalloprotease
APP	Amyloid precursor protein
BACE-1	Beta-secretase 1
BASE	Bovine amyloid spongiform encephalopathy
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CH1641	Chandler strain of scrapie
CNS	Central nervous system
CO_2	Carbon dioxide
CJD	Creutzfeldt Jakob disease
^{Ctm}PrP	Carboxy-terminally attached PrP
CWD	Chronic wasting disease
DEFLIA	Differential extraction lanthanide fluorometric immunoassay
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DIMs	Detergent insoluble microdomains
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylenediaminetetracetic acid
ER	Endoplasmic reticulum
Eu^{3+}	Europium
FCS	Foetal calf serum
fCJD	Familial Creutzfeldt Jakob disease
FDCs	Follicular dendritic cells
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy

G1	PrP glycosylation deficient transgenic, lacking the first N-glycan attachment site (N180T)
G2	PrP glycosylation deficient transgenic, lacking the second N-glycan attachment site (N196T)
G3	PrP glycoylation deficient transgenic, lacking both N-glycan attachement sites (N180T, N196T)
GALT	Gut Associated Lymphoid Tissues
GPI	Glycosyl-phosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ic	Intracerebral
ip	Intraperitoneal
iCJD	Iatrogenic Creutzfeldt Jakob disease
IHC	Immunohistochemistry
kDa	Kilo Dalton
LDS	laemmli sample buffer
LN_2	Liquid nitrogen
LRS	Lympho-reticular system
MAb	Monoclonal antibody
mRNA	Messenger RNA
MBM	Meat and bone meal
MTM	Mixed thymocyte media
NEM	N-Ethylmaleimide
NtmPrP	Amino-terminally attached PrP
NP40	Nonident P40
NPD	Neuropathogenesis Division
NSP	National Scrapie Plan
Oligo	Oligonucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBST	PBS 0.1% Tween
PK	Proteinase K
PMCA	Protein misfolding cyclic amplification
PMSF	Phenylmethylsulfonyl fluoride
PNGase	Peptide N-glycosidase F
PrP	Prion protein
Prions	Proteinaceous infectious particles
PRNP	Gene encoding the human prion protein
Prn-p	Gene encoding the murine prion protein
Prnd	Gene encoding doppel
PrP^C	PrP- cellular; native, uninfectious form of PrP
PrP^d	Disease-associated prion protein (Term used in microscopy)
PrP^{Sen}	Protease sensitive form of the prion protein
PrP^{Sc}	PrP- scrapie; infectious form of PrP
PVDF	Polyvinylidine fluoride
(rPrP)	Recombinant PrP
REML	Residual estimate maximum likelihood
RPMI	Roswell Park Memorial Institute
SCID	Severe combined immunodeficient mice
sCJD	Sporadic Creutzfeldt Jakob disease
^{Sec}PrP	Secretory PrP
SDS	Sodium dodecyl sulphate
Sho	Shadoo protein
SNPs	Single nucleotide polymorphisms
Sprn	Gene encoding Shadoo
SSBP/1	Scrapie brain pool 1
STE	Stop transfer effector region
TBS	Tris buffered saline
TBST	TBS 1% Tween
TACE	Tumor necrosis factor α-converting enzyme

Tg	Transgenic
TME	Transmissible mink encephalopathy
TM1	Transmembrane region 1
TSE	Transmissible spongiform encephalopathy
UV	Ultraviolet light
vCJD	Variant Creutzfeldt Jakob disease
w/v	Weight/volume
WT	Wild type

Figure list

Tables

Chapter 1 Introduction

1.1 Transmissible spongiform encephalopathies

1.1.1 General Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of fatal infectious neurodegenerative diseases that affect both humans and animals. Examples of prion diseases include bovine spongiform encephalopathy (BSE), scrapie, chronic wasting disease (CWD) and Creutzfeldt-Jakob disease (CJD) (Table 1.1). TSEs are associated with degeneration of the central nervous system (CNS) that can be observed histologically by vacuolation of neuronal cell bodies, astrocytic gliosis, reduction in neural tissue and sometimes the presence of amyloid within the brain. Common clinical signs observed with human prion diseases include ataxia, dizziness, and at later stages cognitive deficits. In scrapie, behavioural changes are followed by pruritus that causes sheep to scrape, which results in wool loss.

The abnormal form of the prion protein is referred to as PrP^{Sc} (^{Sc} for scrapie) (Prusiner, 1982). PrP^{Sc} can be distinguished from the normal cellular form of the prion protein (PrP^{C}), as PrP^{Sc} is partially resistance to proteinase K (PK) digestion. PrP^{res} (^{res} for resistant) is the term used to describe proteinase K resistant forms of PrP. There are also abnormal forms of PrP that are sensitive to Proteinase K digestion; these forms of PrP are referred to as PrP^{sen} (^{sen} for sensitive). In microscopy abnormal forms of PrP are referred to PrP^{d} since protease digestion is often not tested.

There have been cases where the levels of abnormal PrP do not correlate with infectivity titre and mouse/TSE strain combinations have been identified that show little quantitative association with abnormal PrP deposition (Lasmezas *et al.*, 1997, Baron *et al.*, 2007, Piccardo *et al.*, 2007).

Ovine PrP^{C} protein consists of 256 amino acids (Figure 1.1); PrP^{C} in other mammalian species is of similar length. The structure of the prion protein was

determined by nuclear magnetic resonance (NMR). The NMR analysis showed that the prion protein contained two structural domains; an N-terminal region of around 100 amino acids which is highly flexible and unstructured, and a globular highly structured C-terminal region (Riek *et al*., 1996, 1997, Zahn *et al*., 2000, Lysek *et al*., 2005). The N-terminal domain of PrP contains the signal peptide and the octapeptide repeat region (PHGGGWGQ). The number of repeats within the octapeptide region can vary among species. Within the octapeptide repeat region cleavage (β cleavage) occurs at amino acid 90 which results in the formation of two fragments, C2 and N2 which are 21 kDa and 7 kDa in size.

The globular C-terminal domain of PrP is important for membrane attachment via a glycophosphotidylinositol (GPI) anchor and is arranged in three α-helices corresponding to amino acids 147-157, 176-197 and 203-231 (ovine PrP numbering) and two anti-parallel β-strands forming a small sheet. The C-terminal domain of PrP also contains a highly conserved hydrophobic domain which contains a transmembrane region. Within the hydrophobic region another cleavage event occurs at amino acid 115/116 which results in the formation of two fragments, C1 and N2 which are 18 kDa and 9 kDa in size. A disulphide bond between cysteine residues at amino acid 179 and 214 stabilises the strucuture of the prion protein (Linden *et al*., 2008) whereas two N-linked glycosylation sites at asparagines 181 and 197 give rise to diglycosylated and monoglycosylated forms of PrP.

The conversion of PrP[C] to PrP[Sc] during TSE infection is not fully understood but involves a shift from predominantly alpha helical structure to a structure rich in beta sheet. The beta sheet structure allows the protein to form oligomers and eventually aggregates that accumulate in the brains of TSE infected animals, sometimes as amyloid fibrils/plaques. Upon Proteinase K digestion PrP[Sc] loses residues 23-89 and leaves behind a hydrophobic, protease resistant core. Proteinase K is added to brain homogenate prior to immunodetection as this protease degrades the normal cellular prion protein.

There is some evidence to suggest that increased concentration of PrP^C at the cell membrane can cause the protein to form dimmers which can lead to the formation of beta sheet structure (Elfrink *et al*., 2008). In a separate study incubation of recombinant PrP under acidic conditions, in the presence of guanidine hydrochloride resulted in an increase in β-sheet structure (Zhou, 2009) of recombinant PrP. Two models of prion conversion have been proposed, the nucleated polymerisation seeding model and refolding model. In the nucleated polymerisation seeding model the conformational change of PrP^C to PrP^{Sc} is thermodynamically controlled, and under equilibrium PrP^C conformation is favoured. Only when aggregates of PrP^{Sc} are added is PrP^C able to convert to PrP^{Sc}. In the refolding model it is proposed that PrP^C and PrP^{Sc} form heterodimers that go on to form homodimers of PrP^{Sc}.

TSEs can occur sporadically, may be inherited genetically in humans and other animals through mutations in the *PRNP* gene (*PRNP, prn-p*), or can be acquired iatrogenically. TSEs are special, in terms of protein misfolding diseases because of their infectious nature (Parchi *et al*., 2000). TSEs can also be experimentally transmitted to animals as was first demonstrated by Cuille & Chelle in 1939. Throughout this thesis PrP^{Sc} will be used to describe the disease associated form of PrP^C; which is the normal cellular form.
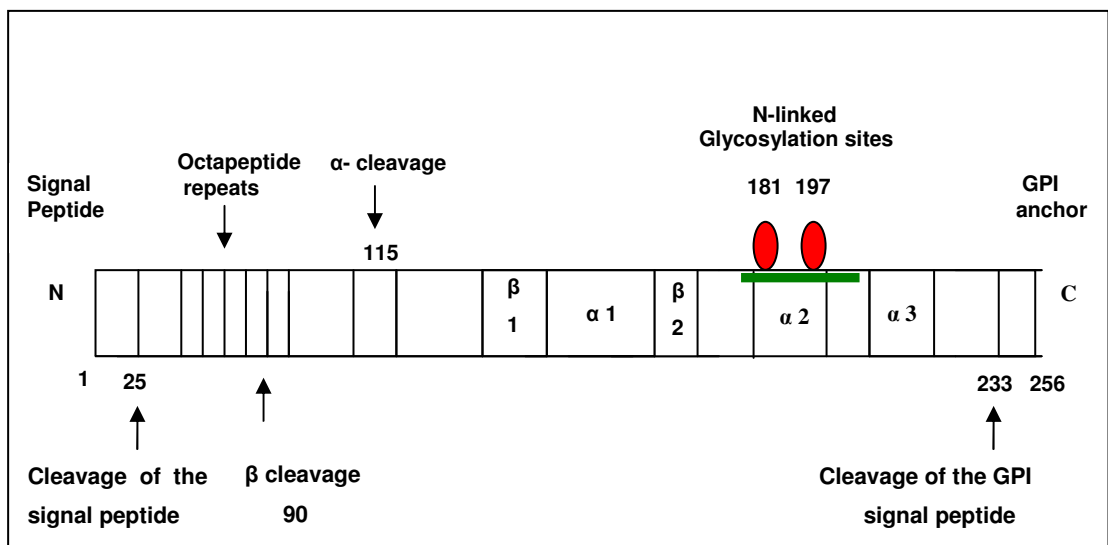
Figure 1.1 Structure of Ovine PrP^C

Linear representation of ovine PrP^C prior to cleavage of the N-terminal signal peptide (1-25) and C terminal GPI-signal peptide (233-256) including the α and β cleavage sites, glyscosylation sites at amino acid 181 and 197 (Highlighted in red), octapeptide repeat region and the position of the α helixes and β sheets. Disulphide linkages between cysteine residues at amino acid 179 and 214 help to stabilise the structure of the prion protein (area highlighted in green).

Table 1.1 Transmissible spongiform encephalopathies

Natural Host	Disease	Aetiology	References
Sheep/ Goats	Classical scrapie	Infectious	(Cuille & Chelle, 1939, Pattison & Millson, 1960)
	Atypical scrapie	Unknown	(Benestad *et al.*, 2003, Hunter, 2007, Nentwig *et al.*, 2007) review (Benestad *et al.*, 2008)
Mule deer and Elk	Chronic wasting disease (CWD)	Infectious	(Williams & Young, 1980,1982)
Cattle	Bovine spongiform encephalopathy (BSE)	Contaminated feed	(Wells *et al.*, 1987)
Mink	Transmissible mink encephalopathy (TME)	Idiopathic	(Hartsough & Burger, 1965, Kimberlin & Marsh, 1975)
Cats	Feline spongiform encephalopathy (FSE)	Contaminated meat	(Wyatt *et al.*, 1991)
Human	CJD (sCJD)	Idiopathic	
	CJD	Iatrogenic	Review (Prusiner, 1993, Wadsworth & Collinge, 2007)
	CJD (fCJD)	Inherited and associated with *PRNP* mutations	
	vCJD	Consumption of BSE contaminated food	(Bruce *et al.*, 1997, Will *et al.*, 1996)
	Gerstmann-Sträussler-Scheinker Syndrome (GSS)	Inherited and associated with *PRNP* mutations	(Ghetti *et al.*, 1994)
	Fatal familial insomnia (FFI)	Inherited and associated with *PRNP* mutations	(Will *et al.*, 1996)
	Kuru	Ritual cannibalism	(Gajdusek & Zigas, 1957) * review (Wadsworth *et al.*, 2008)

1.1.2 Scrapie

Classical scrapie is an infectious TSE of sheep and goats (Parry, 1962). The disease is endemic in many countries with the exception of New Zealand and Australia which are thought to be free of classical scrapie. In the United Kingdom, scrapie is a notifiable disease. Since 1993 any animal suspected of having scrapie must be reported to the local Animal Health Divisional Office (AHDO). Sheep that are positive for scrapie must be culled, as the disease could spread to other sheep within the flock.

Clinical signs that are observed in scrapie infected sheep and goats include increased excitability, aggression, depression, incoordination, skin irritation, weight loss and eventually death (McGowan, 1922, Parry, 1962, Wood *et al.*, 1992, Capucchio *et al.*, 2001, Houston & Gravenor, 2003). *Post-mortem* histopathological examination of the CNS of scrapie infected sheep shows astrocytosis, neurodegeneration with vacuolation of neurones and spongiform changes, which are characteristic of TSEs (Bruce & Fraser, 1975, Jeffrey & Gonzalez, 2007). Like for all TSEs there are no pre-mortem diagnostic tests available for scrapie, so detection is usually carried out on *post-mortem* tissue. PrP^{Sc} is detected from tissue by immunohistochemistry (IHC) or Western blot after treatment with proteinase K. Proteinase K is added to the tissue prior to immunodetection as this protease degrades the normal PrP protein (PrP^{C}) leaving behind only the disease associated protease resistant PrP^{Sc} protein.

Scrapie was first experimentally transmitted to sheep by intra-ocular administration of a scrapie infected brain homogenate Cuille & Chelle (1939). Since then scrapie has been successfully experimentally transmitted to sheep, goats, cattle, mice, rats and hamsters. In sheep the route of transmission for natural scrapie is unclear, both horizontal (between sheep) (Brotherston *et al.*, 1968) and vertical transmission (between ewe and the lamb) (Dickinson *et al.*, 1974) have been suggested.

Evidence for horizontal transmission comes from studies that show that healthy "scrapie free" sheep can develop disease when they are housed in contact with scrapie infected sheep (Foster *et al.*, 2006). Earliest evidence for vertical

6

transmission comes from Dickinson *et al*., (1965) who observed that lambs born from infected ewes were more likely to develop disease than lambs born to non-infected mothers. More recent embryo transfer studies have shown vertical transmission in sheep; those results however did not allow an assessment of the efficiency of this mode of transmission (Foster *et al*., 1992, Wang *et al*., 2001, Wrathall *et al*., 2008).

Oral route of transmission has been favoured as the main candidate in natural scrapie cases (Kimberlin & Walker, 1989a) although scrapie can also be transmitted through skin abrasions (Taylor *et al*., 1996) and through infected milk (Konold *et al*., 2008). Other routes of transmission may also be possible. For e.g. transmission may occur through the environment by soil contaminated with infected urine (Seeger *et al*., 2005, Siso *et al*., 2006, Andrievskaia *et al*., 2008) or faeces (Georgsson *et al*., 2006, Johnson *et al*., 2006). Evidence of scrapie infection in the placenta has also been investigated (Race *et al*., 1998). Most recently scrapie infection has been shown to occur after infected donor blood has been transfused to an uninfected animal (Houston *et al*., 2008, Siso *et al*., 2010).

1.1.3 Atypical scrapie

The first cases of atypical scrapie were identified in Norway in 1998 (Benestad *et al*., 2003). These cases were distinct from classical scrapie and were designated Nor98. The most common clinical sign in atypical scrapie is ataxia (Benestad *et al*., 2003). Vacuolation and PrP^{Sc} deposition is found more commonly within the cerebellar region of the brain (Benestad *et al*., 2003, Hunter, 2007, Nentwig *et al*., 2007). Atypical scrapie is also distinct from classical scrapie when PrP^{Sc} is analysed by Western blot (Figure 1.2).

Atypical scrapie has been successfully transmitted to sheep (Simmons *et al*., 2007) and transgenic (Tg) mice (Tg 338) that over-express ovine PrP^{C} (Le Dur *et al*., 2005), providing evidence that atypical scrapie is a true prion disease. It has been proposed that atypical scrapie may be a sporadic disease as these cases occur in small

numbers within sheep flocks with no indication of horizontal or vertical transmission (Benestad *et al.*, 2003, Hopp *et al.*, 2006).
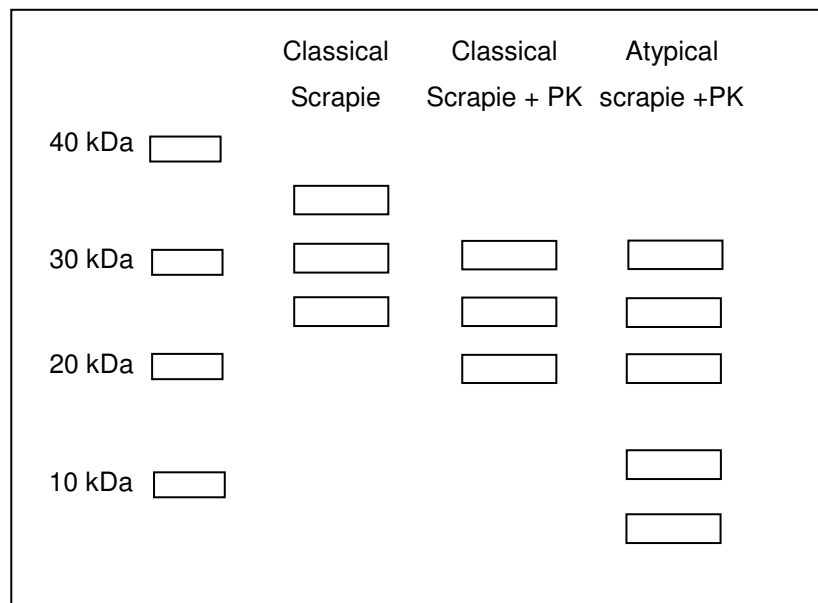
Figure 1.2 Expected Western blot PrP^{Sc} banding patterns and molecular weight distribution in classical and atypical scrapie

Approximation of molecular weight for classical and atypical scrapie with PK treatment on SDS PAGE. For classical scrapie without treatment with PK the di-glycosylated protein is detectable at 35-36 kDa, monoglycosylated protein at 30-32 kDa and unglycosylated protein at 25-27 kDa. For classical scrapie with treatment with PK the di-glycosylated protein is detectable at 26-30 kDa, mono-glycosylated protein at 23-25 kDa and unglycosylated protein at 20-22 kDa. Atypical forms have also been reported to have additional bands at 12 kDa and 7 kDa (Gretzschel *et al.*, 2006).

1.1.4 TSE and ovine PrP polymorphisms

A major factor that has been associated with susceptibility to TSE disease is *PRNP* genetics. The most common type of polymorphism found in *PRNP* is the single nucleotide polymorphism (SNP). SNPs can produce either a missense mutation (amino acid replaced with a different amino acid) or a silent mutation (amino acid remains the same). The other type of polymorphisms found in PrP are insertions or deletions within the octapeptide repeat region of the N-terminal domain of PrP. In most species there are five copies of an eight amino acid sequence PHGGGWGQ (octapeptide). However, in ruminants this number can vary between three and seven copies (Goldmann *et al*., 1991b,1998, Schlapfer *et al*., 1999).

Polymorphisms located at 136 [valine (V) or alanine (A)], 154 [arginine (R) or histidine (H)] and 171 [glutamine (Q), (H) or (R)] are associated with susceptibility or resistance to natural and experimental scrapie (classical scrapie) (Figure 1.3) and produce five common alleles: ARR, ARQ, AHQ, ARH and VRQ.

Evidence for a linkage between codon 136 and disease susceptibility was discovered after sheep carrying the valine allele at codon 136 were experimentally challenged with scrapie (Goldmann *et al*., 1991a). Following inoculation with scrapie brain homogenate SSBP/1, homozygous animals for V136 had a mean incubation period of 168 days compared to a mean incubation period of 309 days for heterozygous animals V136/A136 and A136/A136 sheep survived. Further evidence for a linkage between codon 136 and scrapie disease susceptibility came from epidemiological data from France that showed that all scrapie infected sheep from IIe-de-France and Romanov breeds were either homozygous for valine at 136 (V136/V136) or heterozygous for valine at 136 (V136/A136) (Laplanche *et al*., 1993).

To add complexity to the situation, some breeds which only carry the A136 allele i.e. Suffolk sheep are highly susceptible to scrapie (Westaway *et al*., 1994b). Therefore A136 appears only to provide partial resistance against scrapie and other factors such as breed of sheep and strain of scrapie may influence the effects of *PRNP* genotype on scrapie susceptibility or resistance (Goldmann *et al*., 1994). In terms of disease

the most susceptible genotype to classical scrapie is therefore VRQ/VRQ and the most resistant to classical scrapie is ARR/ARR (Belt *et al*., 1995, Hunter *et al*., 1996).

The genetics of sheep with atypical scrapie are different from the genetics of sheep with classical scrapie. Atypical scrapie cases tend to have genotypes that are associated with resistance to classical scrapie. An association at codon 141 has also been found in these cases, with phenylalanine (F) more commonly found than leucine (L) (Moum *et al*., 2005).

Scrapie is not a genetic disease as susceptible genotypes are found in sheep in Australia and New Zealand that are free from scrapie (Hunter *et al*., 1997, Bossers *et al*., 1999). Also the ARR/ARR homozygotes that are resistant to classical scrapie are susceptible to intracerebral (i.c.) inoculation of BSE (Houston *et al*., 2003) and sheep with susceptible genotypes living in the UK that are reared in clean "scrapie free environments" do not succumb to disease (Foster *et al*., 2006). Heterozygote animals from different breeds also have varying incubation periods (Houston *et al*., 2002a) which suggests that other factors must be involved in disease susceptibility. One of these factors could be the age of the animal as natural infection with scrapie is thought to occur mainly in young sheep (St Rose *et al*., 2007). The biochemistry of the prion protein such as its proteolytic processing could also play a role (Refer to 1.4.7 for further details).

1.1.5 The National Scrapie Plan

In an attempt to control scrapie within the UK sheep population the British Government established the National Scrapie Plan (NSP) in 2001 with an aim to reduce and eventually eradicate scrapie from the national sheep flock by breeding for genetic resistance (DEFRA, 2007). In 2004 the compulsory scrapie flocks scheme was also set up following EU legislation. The National Scrapie Plan was to "initially concentrate on promoting the use of the ARR gene and excluding the VRQ gene and allow the continued use of sheep with the AHQ, ARQ and ARH genes for a limited

period". However, a potential problem has arisen in the past ten years in the form of atypical scrapie that affects animals that are more resistant to classical scrapie.

The NSP and the EU regulations have been successful in that the number of VRQ allele carriers has been reduced significantly. Arnold *et al.*, 2002, Tongue *et al.*, 2008, Melchior *et al.*, 2010, Hagenaars *et al.*, 2010. The breeding schemes in the UK are now voluntary programmes.
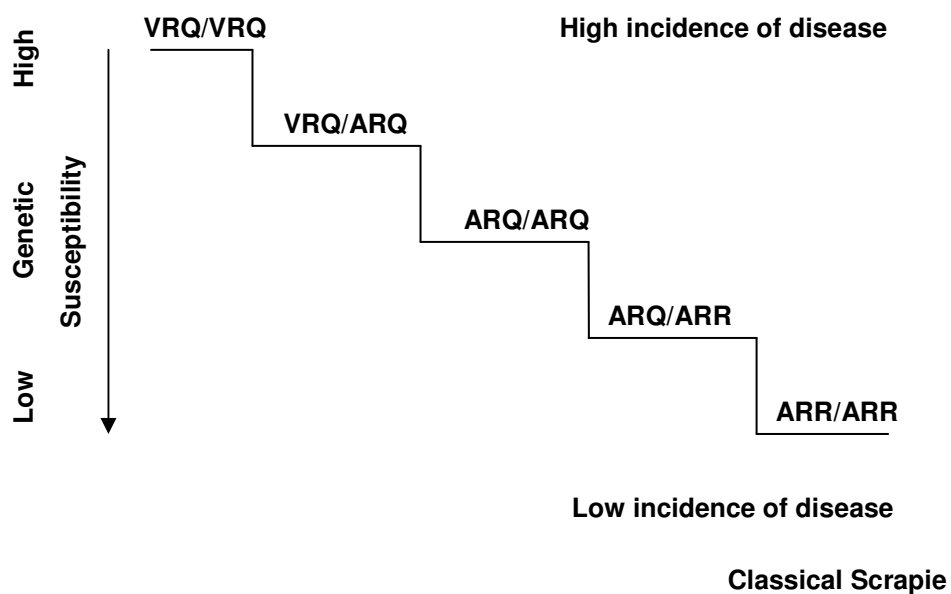
Figure 1.3 Ovine genotypes with different levels of susceptibility to classical scrapie
Polymorphisms in the coding region of PrP have an effect on the susceptibility to scrapie. Animals most susceptible to classical scrapie are homozygous for VRQ and animals most resistant to classical scrapie are homozygous for ARR.

1.1.6 TSE strains and the species barrier

Many isolates from naturally occurring scrapie cases in sheep and goats have been passaged into inbred mice. From these passages approximately twenty different strains of scrapie have been identified (Kimberlin *et al*., 1989). Each new scrapie strain shows unique clinical presentation, neuropathology, PrP^{Sc} deposition pattern, PrP^{Sc} glycoform pattern or incubation period (Fraser & Dickinson, 1967, Somerville, 1999). The pattern of vacuolation within the brain of scrapie infected mice can also be used to distinguish between scrapie strains. Brain areas are scored according to the severity and distribution of the vacuolation and the values are plotted on a graph to form a lesion profile (Fraser & Dickinson, 1968, Bruce & Fraser, 1991).

Some strains can be distinguished by differential PrP glycosylation which gives rise to different glycoform patterns, when observed on SDS-PAGE (Parchi *et al*., 1996). However, two strains or isolates can have identical glycoform patterns. For example the scrapie isolates CH1641 and BSE have been found to have similar banding patterns (Hope *et al*., 1999, Baron *et al*., 2000). These two isolates can be distinguished using Immunohistochemistry (IHC), as PrP^{Sc} staining is intracellular in CH1641 and extracellular in BSE (Jeffrey *et al*., 2006). PrP^{Sc} deposition in the CNS and lympho-reticular system (LRS) of classical scrapie and atypical scrapie also differs. In classical scrapie, PrP^{Sc} deposition is located in the LRS as well as the CNS whereas in atypical scrapie PrP^{Sc} is mainly confined to the CNS (Benestad *et al*., 2003).

Inter-species susceptibility to TSE disease is dependent on many factors including route of inoculation, dose of inoculum, genetic factors but it also appears to be dependent on the sequence homology between species (Scott *et al*., 1993). Transmission of prion infection from one species to another is generally inefficient and is usually accompanied by long incubation periods in the initial passage; this effect is known as the species barrier. In some cases this barrier can be overcome by secondary passage into the same species, this usually results in shortened incubation periods (Pattison, 1966, Dickinson, 1976). The species barrier can also be overcome

through the use of transgenic mice that express the PrP transgene identical to the infected donor. For example wild type mice are resistant to TSE disease after challenge with hamster scrapie, while transgenic mice that express hamster PrP are susceptible to this challenge (Scott *et al*., 1989,1993, Race *et al*., 1995). This indicates that the primary sequence is important in the susceptibility to TSE disease.

In some cases the species barrier is breached; this has been shown in the ability of BSE to infect cats (Wells *et al*., 1987, Wyatt *et al*., 1991) and humans (Will *et al*., 1996). The bank vole is an unusual model as it appears to be susceptible to many TSEs even when there is low PrP sequence homology between the species (Cartoni *et al*., 2005, Nonno *et al*., 2006, Agrimi *et al*., 2008). In other cases it may not be possible for the species barrier to be crossed; for example there have been no reported cases of sheep scrapie transmission to humans or any reports of TSE disease in dogs.

1.1.7 TSE pathogenesis

The site of entry, accumulation and replication of the prion agent within the body appears to vary between species (Mabbott & MacPherson, 2006). Most natural TSE diseases are thought to occur following exposure to an infectious agent. In sheep infected with classical scrapie, PrP^{Sc} usually accumulates and replicates within the LRS (van Keulen *et al*., 1996, Andreoletti *et al*., 2000) before spreading to the CNS. As PrP^C is expressed widely throughout the body this protein does not produce a specific immunological response to the TSE agent (Porter *et al*., 1973). However, activation of microglial cells and astrocytes has been observed during the disease process (Prusiner, 1994, Crozet *et al*., 2008).

In classical scrapie cases PrP^{Sc} has been detected in gut associated lymphoid tissues (GALT) and Peyer's patches (van Keulen *et al*., 1996, Andreoletti *et al*., 2000) as well as central nervous tissue. Soon after experimental inoculation with scrapie, there is rapid accumulation of PrP^{Sc} in the spleen which is detectable weeks or even months before detection of PrP^{Sc} in the CNS. Studies have shown that the peripheral

system plays an important role in the pathogenesis of some TSEs as the removal of the Peyer's patches and the spleen in mice infected with the scrapie agent increases the incubation period and the time which it takes infectivity to reach the CNS (Fraser & Dickinson, 1970, Prinz *et al*., 2003).

Scrapie susceptibility is also reduced in B cell deficient mice and in severe combined immunodeficient mice (SCID) that lack T and B lymphocytes (Fraser *et al*., 1996) as well as follicular dendritic cells (FDCs) (Brown *et al*., 1999). SCID mice are resistant to scrapie infection following peripheral exposure to low doses of inoculum, however grafting of bone marrow from wild type donors restores susceptibility to disease. It has been shown that early accumulation of PrP^{Sc} occurs on FDCs (Brown *et al*., 1999, Mabbott *et al*., 2000) which normally expresses high levels of PrP^{C} on their cell surface (McBride *et al*., 1992, Mabbott *et al*., 2000).

However, not all TSE diseases accumulate PrP^{Sc} within the LRS: for example, PrP^{Sc} in cattle BSE appears to be confined to the CNS (Terry *et al*., 2003, Iwata *et al*., 2006, van Keulen *et al*., 2008). This indicates that the TSE agent is able to bypass the LRS on the way to the CNS. The most direct route for this would be through the peripheral nerves (Beekes & McBride, 2007). Once the agent reaches the CNS it appears to target neurones and in particular the GABAergic neurones (Guentchev *et al*., 1998). Generally the most commonly observed neuronal changes associated with TSE disease are loss of synapses, dendritic atrophy and neuronal loss (Giese *et al*., 1995, Lucassen *et al*., 1995, Williams *et al*., 1997, Fraser, 2002, Unterberger *et al*., 2005, Kovacs & Budka, 2008).

1.1.8 The effect of age on TSE susceptibility

The effect of age on TSE susceptibility was first noted when neonatal mice were found to be partially resistant to peripheral infection with doses that normally produced disease in adult mice. The incubation period of the neonatal mice that did succumb to disease was also longer than adult mice (Outram *et al*., 1973, Ierna *et al*., 2006). The reason behind this age phenomenon was attributed to lack of spleen development in the neonatal mice (Ierna *et al*., 2006).

Susceptibility to TSE disease in other species also appears to be age dependent, for example most cattle that developed BSE were infected as calves (Arnold & Wilesmith, 2004) and young lambs have been shown to be more susceptible than adults to natural infection with scrapie (Elsen *et al*., 1999). An age related susceptibilities also appears to be important in vCJD as clinical disease occurs predominantly in young adults (Ghani *et al*., 2003) although the reasons behind this are unknown.

As animals age there is a dramatic decline in immune function, this is partially due to a reduction in T cell production which makes older animals more susceptible to infection with various diseases. (Maue, 2008). However, in terms of TSE disease it has been shown that sheep first exposed to infection at an older age are less susceptible to scrapie than animals infected at early ages (Matthews *et al*., 2001, Diaz *et al*., 2005, St Rose *et al*., 2007). It has been shown by Brown *et al*., (2009) that there is a decline in the follicular dendritic network in aged mice, which impairs TSE accumulation and neuroinvasion. None of the mice in this study developed clinical TSE disease although histopathological signs of the disease were observed in the brains of these animals (Brown *et al*., 2009). If this phenomenon occurs in humans then this might explain why vCJD has not been observed in older adults who were exposed to BSE by dietary route. These studies collectively illustrate that the age of the animal is an important factor in TSE susceptibility.

1.2 The TSE agent

1.2.1 Identifying and understanding the infectious agent

One of the most debated areas of TSE research is identifying and understanding the infectious agent that causes disease. TSE diseases were initially thought to involve a slow virus (Sigurdsson *et al*., 1957, Hadlow, 1959) because of the long incubation periods associated with the agent. However, studies carried out by Alper *et al*., (1966, 1967) showed that the scrapie agent was extremely resistant to treatments that normally destroy nucleic acids, including UV and ionizing radiation and that the molecular weight necessary for infectivity was so small that it excluded any known

viruses. The work by Griffith, (1967) suggested that the TSE agent could consist solely of a protein. This idea was supported by observations that showed that the agent was sensitive to treatments that denature and degrade proteins (Bolton *et al*., 1982, McKinley *et al*., 1983). From these experiments two hypotheses were produced to explain the nature of the infectious agent.

1.2.2 The prion hypothesis

In 1982 Prusiner hypothesised that the infectious agent that causes scrapie consists of a novel class of particles which he termed prion (proteinaceous infectious particles) (Prusiner, 1992). This hypothesis was based on experimental work (Bolton *et al*., 1982, Prusiner, 1982) that involved the purification and isolation of a partially protease resistant protein from the brains of scrapie-infected hamsters. The protein that was isolated was named PrP^{Sc} (^{Sc} for scrapie). PrP^{Sc} is thought to be the infectious agent in TSE disease or a component of it, as the presence of PrP^{Sc} usually co-purifies with infectivity. However, there have been individual cases where PrP^{Sc} has been present with no infectivity (Piccardo *et al*., 2007) and cases where PrP^{Sc} has been absent in the presence of infectivity (Lasmezas *et al*., 1997).

The prion hypothesis also does not fully explain why PrP^{Sc} with identical primary sequence can give rise to different agent strains, for example the mouse ME7, 22A and 22C (Kimberlin *et al*., 1989). These reproducible and inherited characteristics are conventionally thought to be the preserve of a nucleic acid as different strains of bacteria and viruses arise due to variability in their nucleic acid genomes. To overcome the issues of reproducible TSE strains it has been suggested that PrP^{Sc} from different strains may exist in different conformations (Prusiner, 1998) that can replicate using host PrP^{C} (Soto & Castilla, 2004).

To confirm whether the prion protein is essential for scrapie transmission transgenic mice were created that did not express the PrP protein (*Prn-p*$^{0/0}$). When these mice were challenged with scrapie they did not succumb to disease and appeared to develop normally with no apparent phenotype. When transgenic mice that expressed only one copy of the PrP gene (*Prn-p*$^{0/+}$) were challenged with scrapie they showed

an increase in incubation period compared to wild type mice (Manson *et al*., 1994, Weissmann *et al*., 1994). These experiments do not prove that PrP alone is sufficient for TSE development, only that it is necessary for disease propagation and infectivity. When PrP is over-expressed the incubation time decreases and there is increased PrP^{Sc} formation (Prusiner *et al*., 1990,1998). Certain mutations in the PrP gene are linked to inherited forms of prion disease, which also provides support for the role of PrP^{Sc} in disease pathogenesis (Soto & Castilla, 2004). Other evidence in support for the prion hypothesis is that prions created *in vitro* can be infectious when used *in vivo* (Legname *et al*., 2004, Castilla *et al*., 2005).

1.2.3 The virino hypothesis

The virino hypothesis speculates that the infectious agent consists of an informational component (i.e. nucleic acid) which may interact or be coated with a host-encoded protein; most likely to be PrP^{C} (Dickinson & Outram, 1988, Farquhar *et al*., 1998, Aguzzi *et al*., 2007). This host encoded protein would allow the informational component to reside undetected by the host immune system; this would help explain why there is a lack of immunological response to TSEs. The existence of a nucleic acid would help explain the existence of TSE strains as viral nucleic acids can carry strain specific information (Dickinson & Outram, 1988). Viral like structures have been found in TSE affected brains (Diringer *et al*., 1997) but to date no evidence of a nucleic acid has been found, although this may be because the molecule involved is too small to be detected (Bruce & Dickinson, 1987). In conclusion, both the prion hypothesis and the virino hypothesis do not fully explain the nature of the TSE agent although the prion hypothesis is more universally accepted.

1.2.4 Conversion models

Two models have been proposed for the conversion of PrP^{C} to PrP^{Sc} based on the prion hypothesis; the refolding model (Prusiner, 1991) and the nucleated polymerisation seeding model (Come *et al*., 1993, Jarrett & Lansbury, 1993). The nucleated polymerisation seeding model proposes that under normal circumstances PrP^{C} and PrP^{Sc} are in equilibrium with each other, with PrP^{C} being the most

abundant. Aggregates of PrP^{Sc} can break off which can facilitate the seeding of more PrP^{Sc}. The conformational model proposes that PrP^C interaction with PrP^{Sc} triggers the conversion of PrP^C to PrP^{Sc}. Once PrP^{Sc} has interacted with PrP^C it is then able to convert more PrP^C into PrP^{Sc} by an unknown mechanism that creates a continuous chain reaction.

Conversion of recombinant PrP^C to PrP^{Sc} has been demonstrated *in vitro* (Kocisko *et al.*, 1994, Caughey *et al.*, 1995, Castilla *et al.*, 2005) which provides some evidence that information may be transmitted by molecules other than nucleic acid; this provides support for the prion hypothesis. Early studies showed that PrP^{Sc} synthesised *in vitro* was not infectious or transmissible (Caughey *et al.*, 1995, Hill *et al.*, 1999). However, Castilla *et al.*, (2005) showed that they were able to transmit scrapie to hamsters by inoculation of *in vitro* generated forms of the infectious protein through the PMCA method using PrP^{Sc} as a seed for the conversion reaction. It has been shown that additional co-factors such as RNA and proteoglycan molecules may play a role in the conversion of PrP^C to PrP^{Sc} (Deleault *et al.*, 2003, 2007).

1.2.5 Protein misfolding cyclic amplification (PMCA) and the quaking induced conversion assay (QUIC)

The protein misfolding cyclic amplification method was developed by Saborio *et al.*, (2001) with the aim of accelerating prion replication *in vitro*. The PMCA reaction is a cyclic process composed of two phases. During phase one, the sample which contains small amounts of PrP^{Sc} and large amounts of PrP^C are incubated together to form PrP^{Sc} polymers. During the second phase, sonication is used to break the newly formed polymers into smaller pieces to increase the number of particles for the next round of conversion of PrP^C to PrP^{Sc} (Saa *et al.*, 2006; Soto *et al.*, 2002). It has been shown that the addition of proteoglycans and RNA in the PMCA reaction can increase prion protein conversion (Delaut *et al.*, 2003).

Another method that has been developed with the aim of amplifying infectious PrP^{Sc} is the quaking induced conversion assay (QUIC) (Atarashi *et al.*, 2007). In this assay

recombinant (rPrP) is used as a substrate for amplifying PrP^{Sc} and instead of sonication, automated tube shaking is used to induce conversion. The QUIC assay has been able to detect PrP^{Sc} amplification at similar levels to the PMCA assay but with more sensitivity and faster output than the PMCA method (Atarashi *et al*., 2007). As there is currently no pre-diagnostic test available for the detection of PrP^{Sc} either of these assays could be used to detect small amounts of infectivity that may be present in sub-clinical individuals who want to donate blood. However, as the test may take many hours to run both the PMCA and QUIC method would be no good for mass screening.

1.3 The mammalian prion protein family

1.3.1 Ovine *PRNP* Gene Structure

The normal cellular PrP protein (PrP^{C}) and the disease associated form (PrP^{Sc}) are transcribed from the same gene (Goldmann, 1993); this gene is known as the *PrP* gene (*PRNP*). These two forms of the proteins therefore have the same primary amino acid sequence. The length of *PRNP* is approximately 21 kb and depending upon the species the *PRNP* gene contains either two or three exons, of which a single exon codes for the prion protein (Oesch *et al*., 1985, Baylis & Goldmann, 2004, Michalczyk & Ziman, 2007). For all species, the open reading frame (ORF) is found on the last exon of *PRNP*.

Ovine *PRNP* transcripts are produced as both a 4.6 kb and a 2.1 kb mRNA. The 4.6 kb transcript is found predominantly within the brain whereas both transcripts are found at approximately equal levels in the spleen. The smaller sheep transcript is the result of an alternative polyadenylation signal within the 3' UTR (Goldmann *et al*., 1999). The *PRNP* promoter region has four conserved sequence motifs (Westaway *et al*., 1994a), two of which appear to regulate ovine PrP expression (Burgess *et al*., 2009).

1.3.2 *PRNP* gene expression

PrP^C is critical to TSE development with high levels of expression in the brain, intermediate levels in the peripheral tissues and lowest levels found in the liver where PrP expression is almost undetectable. The PrP^C protein is widely expressed in a range of cell types including neurones (Kretzschmar *et al*., 1986, Moya *et al*., 2000, Mironov *et al*., 2003), glial cells (Moser *et al*., 1995) and FDCs (Bruce *et al*., 2000, Mabbott & Bruce, 2001,Glatzel *et al*., 2004).

During scrapie infection *PRNP* mRNA levels have been shown to increase in certain *PRNP* genotypes after infection with scrapie (Austbo *et al*., 2007). This group also showed that PrP mRNA levels within the ileal Peyer's patch follicles increase in susceptible VRQ lambs but not in lambs with more resistant genotypes after oral inoculation with scrapie. The expression of PrP^C within different blood components in sheep is also highest in VRQ homozygotes and lowest in ARR homozygotes (Halliday *et al*., 2005). These results indicate that an increase in mRNA levels may be linked to scrapie susceptibility, although an increase in *PRNP* mRNA levels is not always indicative of an increase in protein expression. In comparison a recent study by Gossner *et al*., (2009) showed that PrP transcripts did not increase after scrapie infection. This group also confirmed that expression levels of PrP transcripts appeared to be influenced by *PRNP* genotype although VRQ animals did not have the highest levels of PrP expression after inoculation with SSBP/1. If incubation periods are affected by PrP expression levels then individuals who become infected with low levels of PrP expression may have extended incubation periods and *vice versa*.

1.3.3 PrP paralogues

Several other genes belong to the prion gene family; the first of these genes is *Prnd* which produces a protein called Doppel (Dpl). Dpl lacks an octapeptide repeat domain but has similarity to the C-terminal domain of PrP. *Prnd* shares with *Prnp* a similar genomic structure. *Prnd* is normally expressed in the testis (Silverman *et al*., 2000, Mo *et al*., 2001) and heart but not in the brain (Moore *et al*., 1999). In one PrP null mouse line with deletions in the ORF and flanking region, mice developed a

syndrome characterised by ataxia, Purkinje cell degeneration and cerebellar atrophy (Sakaguchi *et al*., 1996). The cause of this syndrome was due to a splicing event that allowed *Prnd* to be under the control of the *Prnp* promoter (Sakaguchi *et al*., 1996, Moore & Melton, 1997) this consequently caused inappropriate expression of *Prnd* in the brain. By reintroducing the *Prnp* transgene this syndrome was reversed. A study by Tuzi *et al*., (2002) concluded that Dpl was unlikely to be involved in naturally occurring TSEs as it did not appear to influence TSE disease in their transgenic mouse models.

Another gene that belongs to the prion gene family is *Sprn* which produces a protein called shadoo (Sho). The gene *Sprn* is found in zebrafish, humans and many other mammalian species. Like *Prnp, Sprn* encodes the entire open reading frame within one exon. Sho demonstrates a number of biochemical and cell biological properties which are similarly displayed in PrP^C. These include N-glycosylation, the addition of a GPI-anchor and a cleavage event that results in the formation of truncated fragments (Watts *et al*., 2007). These truncated fragments have yet to be identified *in vivo*. The hydrophobic region of PrP and Sho is well conserved amongst species and may provide an important functional role for the protein.

Sho is thought to provide neuroprotection within the brain and is known to protect neuronal cells from the neurotoxic effects of Dpl and various deleted forms of PrP (Watts *et al*., 2007, Gossner *et al*., 2009). PrP and Sho also share an alanine rich sequence within their hydrophobic region (Figure 1.4). Like PrP, Sho is also expressed in the brain but unlike PrP Sho is expressed at very low levels in peripheral tissues (Watts *et al*., 2007, Gossner *et al*., 2009).

As Sho is a newly discovered member of the prion family there is little information about whether it may play a role in TSE susceptibility, although this is highly likely based on a CJD study by Beck *et al*., (2008). It has been suggested that Sho may interact directly with PrP, this has been shown in co-immunoprecipitation experiments (Jiayu *et al*., 2009) but it is unknown whether this interaction occurs *in vivo* or what relevance this has for TSE susceptibility.

PrP

KPSKPKTNMKHVAG**AAAAGA**VVGGLGGVMLGSAMSRP

RVRPVRPAPRYAGSSVRV**AAAGAAAGAAAG**VAAGLAAGS

Sho

Figure 1.4 Comparison of the hydrophobic region of PrP and Sho proteins

Codons 104-140 of PrP and codons 45-83 of Sho are shown. The bold area highlights the alanine/glycine rich region of the hydrophobic region.

1.4 The normal prion protein, PrP^C

1.4.1 PrP^C function

PrP^C is thought to be involved in many cellular processes, although the major function of PrP^C remains to be clarified. As most PrP^C is located on the cell surface it may be involved in synaptic function (Collinge *et al*., 1994, Manson *et al*., 1995) cell signalling , cell adhesion or uptake of ligands (Collinge, 2001). PrP null mice (*Prnp* $^{0/0}$) that do not express PrP showed no gross abnormal phenotype (Bueler *et al*., 1992, Manson *et al*., 1994). In later studies it was shown that PrP null mice show signs of altered circadian rhythms, abnormal sleep patterns and electrophysiological abnormalities in synaptic function (Collinge *et al*., 1994, Tobler *et al*., 1996, Huber *et al*., 2002, Fuhrmann *et al*., 2006, Powell *et al*., 2008, Le Pichon *et al*., 2009, Singh *et al*., 2009). Loss of sleep is also associated with human FFI (Lugaresi *et al*., 1998) which suggests that certain areas of the brain associated with circadian rhythms may be affected by the loss of PrP^C.

PrP^C has been shown to bind copper within the octapeptide repeat region *in vivo* (Brown *et al*., 1997, Kramer *et al*., 2001). Copper binding studies have also been carried out with synthetic peptides (Hornshaw *et al*., 1995) and with recombinant PrP (Stockel *et al*., 1998). These studies suggest that PrP may function as a copper transport protein for the internalisation of copper ions (Pauly & Harris, 1998) or it may be involved in the cellular response to oxidative stress (Wong *et al*., 2000, Legname *et al*., 2004) as it has been suggested to have an antioxidant activity similar to superoxide dismutase (SOD) (Brown *et al*., 1999). Copper has also been shown to inhibit the *in vitro* conversion of PrP^C into PrP^{Sc} (Liu *et al*., 2008) when it is present at low pH conditions. PrP knockout mice have lower levels of Zn/Cu superoxide dismutase activity than wild type mice, therefore they are unable to accumulate and bind as much copper as PrP expressing mice. Cultured cells derived from PrP-null mice are also more sensitive to oxidative damage (Brown *et al*., 1997).

PrP^C is also thought to be neuroprotective and is known to protect against proapoptotic stimuli and plays a role in cellular antioxidative defence (Klamt *et al*., 2001, Rachidi *et al*., 2003). Evidence for this neuroprotective function comes from *in vivo* studies of transgenic mice with internal deletions mutants in PrP and PrP null mice. It has also been reported that PrP is up regulated in response to ischemic brain injury (Weise *et al*., 2004, Mitsios *et al*., 2007) and that over-expression of PrP can reduce this ischemic injury (Shyu *et al*., 2005), this also supports the role of PrP in neuroprotection.

1.4.2 PrP^C Trafficking and synthesis

After transcription and processing, mature messenger RNA (mRNA) is exported out of the nucleus into the cytoplasm. In the cytoplasm, mRNA binds to ribosomes on the rough endoplasmic reticulum (ER) where it is translated into protein. Within the ER, cleavage of the prion protein signal peptide occurs as well as correct folding of the protein (Figure 1.5). Also within the ER, PrP^C can be synthesised into three topologies, two transmembrane forms and secreted full-length PrP^C which follows the main synthesis pathway.

From the ER reticulum, PrP^C is transported to the Golgi. Within the Golgi several post-translational modifications take place including the addition of a glycosyl phosphatidylinositol (GPI) anchor which is modified with the addition of a sialic acid residue and alterations of the N-linked oligosaccharide chains (Stahl *et al*., 1990). Alpha cleavage of the prion protein is thought to occur as PrP^C cycles between intracellular compartments (possibly the Golgi) and the cell surface. C1 and N1 fragments, produced by alpaha cleavage are transported to the cell surface. The C1 fragment remains attached to the membrane whereas the N1 fragment is released into the cell medium.

At the cell surface PrP^C is localised in detergent insoluble microdomains (DIMs) which contain cholesterol and glycoshpingolipids (Sarnataro *et al*., 2004). When PrP^C is located on these cholesterol-rich domains it interacts with the laminin receptor precursor (LRP) where it undergoes copper mediated endocytosis via a

clathrin coated pit. In addition copper sulphated glycans may also induce internalisation of PrP^C (Collins *et al*., 2004, Campana *et al*., 2005). Certain studies have suggested that the C-terminal signal sequence may help target the proteins to caveolae-like domains within the cell membrane (Harmey *et al*., 1995, Kaneko *et al*., 1997, Peters *et al*., 2003). Cell culture studies have shown that PrP^C is endocytosed with a half life of twenty minutes taking approximately 60 minutes to travel between the cell surface and the endocytic compartments (Shyng *et al*., 1993)

On the cell surface β-cleavage can occur in response to oxidative stress and produces two fragments termed C2 and N2. The majority of cellular PrP^C (>95%) is recycled as full-length protein, a percentage undergoes endoproteolytic cleavage at both N and C terminals, and the rest of the protein is degraded. Cleavage of the GPI anchor also takes place. Following cleavage of the GPI anchor some of the membrane bound PrP^C is released into the extracellular medium (Shyng *et al*., 1993). Finally, degradation of PrP^C is thought to take place via the endoplasmic reticulum associated degradation (ERAD) proteasome pathway (Yedidia *et al*., 2001). A small fraction of PrP^C is thought to be degraded by lysosomes while the large fraction of PrP^C is recycled back to the cell surface. During this recycling some PrP^C may also be secreted into the extra cellular medium where it associates with exosomes.
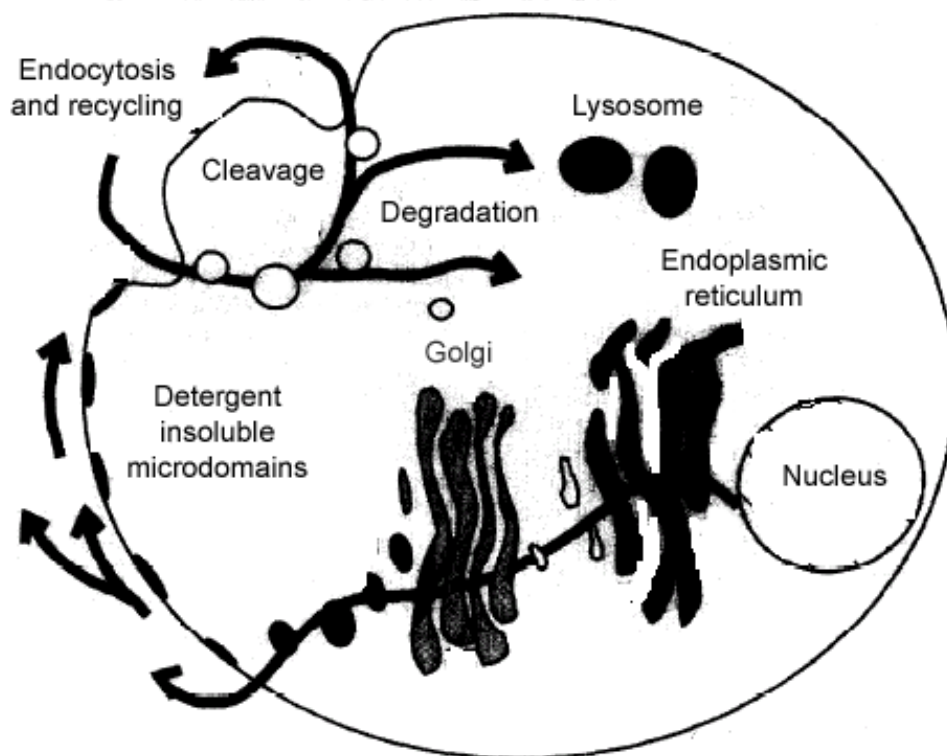
Figure 1.5 General trafficking of PrP^C ($^{©}$ Lehmann *et al.,* 1999)

After transcription and processing, mature messenger RNA is exported out of the nucleus into the cytoplasm. Mature messenger RNA then binds to ribosomes on the rough endoplasmic reticulum where it is translated into protein. In the ER the prion protein signal peptide is cleave and correct folding of the protein takes place.Transmembrane forms of PrP^C are also synthesised within the ER. From the ER reticulum PrP^C is transported to the Golgi. Within the gogli several post-translational modifications take place including the addition of a glycosylphosphatidylinositol (GPI) anchor which is modified with the addition of a sialic acid residues and alterations of the N-linked oligosaccharide chains. Full-length PrP^C can be cleaved within the Golgi (a process called alpha cleavage). The products of this cleavage C1 and N1 are transported to the cell surface where C1 remains anchored and N1 secreted from the cell. PrP^C is integrated into detergent insoluble microdomains (DIM) and sorted. PrP^C is then transported to the cell membrane where it is located on cholesterol rich lipid rafts where it interacts with receptors and copper where it undergoes endocytosis. Once PrP^C is endocytosed it can either be degraded or recycled back to the cell surface.

1.4.3 Glycosylation

Many cell surface glycoproteins have N-linked glycans which are important for protein structure and cellular trafficking (Lowe & Marth, 2003). The prion protein contains two sites for N-glycosylation these sites are located at amino acids 180 and 196 in mice (Oesch *et al*., 1985, Cancellotti *et al*., 2005). The glycosylation sites within the C-terminal domain produce on Western blots three bands of apparent molecular mass between 33 and 35 kDa (Oesch *et al*., 1985). These three bands are interpreted as di-glycosylated PrP (when a carbohydrate is attached to both glycosylation sites), mono-glycosylated (when a carbohydrate is attached to either glycosylation sites) and unglycosylated PrP.

Three lines of gene targeted mice have been produced in which the glycosylation sites have been mutated (Cancellotti *et al*., 2005, Tuzi *et al*., 2008). These three lines of mice are referred to as G1 (N180**T**), G2 (N196**T**) and G3 (N180**T**-N196**T**) (mutations shown in bold letters, numbering refers to murine PrP). G1 and G2 mice lack diglycosylated PrP but express mono and unglycosylated PrP whereas G3 mice only express the unglycosylated form of PrP (Cancellotti *et al*., 2005). *In vivo* analysis of these mice show that the lack of glycans do not influence stability and maturation of PrP however trafficking of PrP^C in the G3 mice appears to be impaired as most PrP was intracellular in these mice. As PrP in G1 and G2 mice were localised to the cell membrane the authors concluded that that only one sugar is required for correct cellular trafficking (Cancellotti *et al*., 2005). Interestingly, the G3 mice did not show any overt phenotype with impaired trafficking. Overall, these results suggest that disease susceptibility may only be partially dependent on glycosylation.

1.4.4 Proteolytic processing of the Alzheimer precursor protein (APP)

In recent years there has been a rising interest in the possibility of an association between TSE disease and Alzheimer's disease (Senior, 2000, Checler & Vincent, 2002, Hooper & Turner, 2008). In both situations abnormal misfolding of a protein leads to neurodegeneration. TSE disease is transmissible to humans and animals; to date there is no evidence that Alzheimer's disease is infectious (Godec *et al*., 1994).

Alzheimer's disease is characterised by the presence of amyloid fibrils, senile plaques and neurofibrillary tangles. In Alzheimer's disease the release of the Aβ peptide is crucial for the development of Alzheimer's disease (Hooper & Turner, 2008). The proteolytic processing of the APP is thought to occur by β and γ secretases within the amyloidogenic pathway which results in the release of the toxic Aβ peptide. Alternative non-amyloidogenic proteolytic processing occurs within the toxic Aβ domain of APP which inhibits the production of Aβ. This alternative cleavage is thought to occur through the action of ADAM (a desintegrin and metalloprotease) proteases.

Proteolytic processing of the prion protein also occurs within a toxic domain (For further details refer to 1.4.7). Cleavage within the PrP toxic domain produces two fragments N1 and C1 and is thought to involve some of the ADAM proteases that are involved in the release of APP (Vincent et al., 2001). Under pathological conditions both APP and PrP^C undergo alternative cleavage events which leave the toxic domains intact (Chen et al., 1995, Vassar, 2001, Checler & Vincent, 2002). The enhancement of these ADAM proteases could therefore provide a therapeutic target for the Alzheimer's and TSE disease.

1.4.5 Transgenic mouse models

Although mice are not a natural host for scrapie, they have been used extensively as a model for TSE pathogenesis to expand our knowledge about PrP expression and disease progression. The benefits of PrP transgenesis was observed when mice expressing Syrian hamster PrP were rendered susceptible to the 237K hamster adapted scrapie strain, in which wild-type counterparts were resistant (Scott et al., 1989). Since this discovery, mice transgenic for the $PRNP$ gene of many different species have been created. Ovine PrP transgenic mice are much easier to maintain and manage than ruminants and have contributed to the current understanding of natural scrapie and atypical scrapie. These ovine PrP transgenic mice are more accurate for studying sheep scrapie pathogenesis than wild type mice as issues such as strain adaptation (Priola, 1999) or failure to elicit a disease response (Hunter, 1997) have been overcome with the introduction of the ovine $PRNP$ sequence. For

example, it has been shown that transgenic mice that over-express ovine PrP^C are more susceptible to disease than transgenic mice that over-express murine PrP^C (Vilotte *et al*., 2001). This indicates that transgenic mice that express ovine PrP^C may be a more useful model for studying the effects of natural scrapie.

In some transgenic models over-expression of the PrP transgene leads to a neurodegenerative disease. In many over-expressing transgenic mouse models shortened incubation times are observed after inoculation with scrapie. For example, TgOvPrP4 mice that express 2-4 fold more PrP compared to a normal sheep have significantly shorter incubation periods compared to wild type mice. Shortened incubation periods were thought to result from a combination of PrP^C over-expression and a reduced species barrier (Crozet *et al*., 2001).

Transgenic mice have also been created that express various truncated forms of PrP (Table 1.2). Mice with deletions at residues 32-121, 32-134 (Shmerling *et al*., 1998), 94-134 (Baumann *et al*., 2007), and 105-125 (Li *et al*., 2007) went on to develop a neurodegenerative condition whereas mice with deletions at residues 32-80, 32-93, 32-106 (Shmerling *et al*., 1998), 108-121 (Muramoto *et al*., 1997) and 114-121 (Baumann *et al*., 2007) did not develop disease. The area of PrP within the central domain of PrP appears to be crucial for disease development in these mice.

Within the central domain of PrP, proteolyitc cleavage of the murine protein occurs at codons 111/112 (alpha cleavage), this produces two truncated fragments termed C1 and N1. In all of the mice with deletions that went on to develop disease the alpha cleavage site was also disrupted except for deletion 108-121 and in mice that did not develop disease the alpha cleavage site was intact. This suggests that the function of this cleavage event may be important factor in neuroprotection. (This is discussed in relation to PrP in 1.4.7). With the introduction of the wild type PrP gene the symptoms of these animals were reversed. This evidence supports the idea that PrP^C provides a protective role against neurodegeneration.

In another study Muramoto *et al*., (1997) showed that mice with deletions within the alpha helix region of PrP (177-190 and 201-217) went on to develop a neurological disease. This region of PrP may be critical for correct conformation of the prion protein. These mice showed different clinical phenotypes to mice that had deletions within the central domain of PrP (Table 1.2).

In conclusion, studies that have used deleted forms of PrP (Muramoto *et al*., 1997, Shmerling *et al*., 1998, Baumann *et al*., 2007, Li *et al*., 2007) have shown that particular areas of the prion protein appear to be critical in maintaining essential biological function.

Table 1.2 Effects of deletions within PrP (adapted from Linden *et al.*, 2008)

Deletion Δ	Neurodegeneration	Reintroduction of PrP[C]
32 - 80[1]	No	Not required
32 - 93[1]	No	Not required
32 - 106[1]	No	Not required
32 - 121[1]	Yes-demyelination and axonal loss	Yes- reversal of abnormal phenotype
32 - 134[1]	Yes-demyelination and axonal loss	Yes-reversal of abnormal phenotype
94 - 134[2]	Yes-demyelination ataxia, astrocytosis	Yes-reversal of abnormal phenotype
105 - 125[3]	Yes- cerebral atrophy death within 1 month of birth	Yes-reversal of abnormal phenotype
108 -121[4]	No	Not required
114 – 121[2]	No	Not required
177 – 200[4]	Yes - loss of axons in white matter	Not carried out in study
201 – 217[4]	Yes – extensive nerve cell loss in CA1 of hippocampus	Not carried out in study

[1](Shmerling *et al.*, 1998) [2](Baumann *et al.*, 2007) [3](Li *et al.*, 2007) [4] (Muramoto *et al.*, 1997) Deletion mutants of PrP and the phenotypes that are produced are shown above. If there is any neurodegeneration associated with these mutants reintroduction of wild type PrP appears to reverse the abnormal phenotype.

1.4.6 Transmembrane forms of PrP

Several alternative forms of PrP^C distinct from GPI-anchored PrP^C and disease associated PrP^{Sc} have also been proposed as key pathogenic entities in prion disease (Hegde *et al*., 1998a, Stewart & Harris, 2005). Two transmembrane forms of PrP^C exist; (^{Ctm}PrP) and (^{Ntm}PrP) which span the membrane at amino acids 113-135 in the area termed transmembrane region or TM1 (Brockes, 1999). The C-terminus of ^{Ctm}PrP and the N-terminus of ^{Ntm}PrP is situated within the ER lumen (Hegde *et al*., 1998b, Stewart & Harris, 2001). However, in it has been shown that ^{Ctm}PrP does not remain in the ER and localises to the Golgi (Stewart & Harris, 2005).

Transmembrane forms of PrP are thought to arise when PrP signals fail to initiate translocation (Ranee *et al*., 2010). This results in either the release of cytosolic PrP, which is degraded in the proteasome or ^{ctm}PrP and ^{ntm}PrP depending on the orientation in the ER lumen. The synthesis of these transmembrane forms within the ER is dependent on prion protein sequence 104-135, as it has been shown that mutations, deletions and insertions within this region can alter the amount of transmembrane PrP that is synthesised (Hedge *et al*., 1998).

^{Ctm}PrP has been implicated in prion disease pathogenesis as transgenic mice expressing ^{Ctm}PrP mutations develop a spontaneous neurodegenerative illness similar to prion disease but without detectable levels of PrP^{Sc} (Hegde *et al*., 1998a, Stewart & Harris, 2005) and it is usually not transmissible (Hegde *et al*., 1998b). An increase of ^{Ctm}PrP has also been observed in some patients with the A117V mutation in GSS disease and in mice expressing a PrP transgene with the A117V mutation (Hegde *et al*., 1998a, Stewart & Harris, 2001).

1.4.7 PrP^C cleavage

PrP^C usually exists as a membrane attached glycoprotein but minor fragmentss can span the membrane and be released as soluble PrP^C. During post-translational modification of the protein, specific cleavage (alpha cleavage) of full-length PrP occurs within the hydrophobic "toxic" region (106-126) of the prion protein, C-

terminal to amino acid 115 (Chen *et al*., 1995, Mange *et al*., 2004, Tveit *et al*., 2005). PrP 106-126 is rich in beta sheet structure, forms aggregates that are detergent-insoluble and PK resistant and is toxic to primary cultures of Neurones (Ettaiche *et al*., 2000, Haik *et al*., 2000).

Alpha cleavage of the prion protein can substantially reduce the level of full-length prion protein and generates PrP fragments with a molecular mass of 9 kDa (N1) and 18 kDa (C1), respectively (Harris *et al.*, 1993, Chen *et al*., 1995, Jimenez-Huete *et al*., 1998) (Figure 1.6 and 1.7). The C1 fragment is attached to the plasma membrane via a GPI anchor whilst the N1 fragment is realeased from the membrane by shedding (Vincent *et al*., 2000). Both fragments appear to have some function in normal cell biology, which is not fully understood (Haigh *et al*., 2010). Their involvement in scrapie disease is also still unclear. It is possible that alpha cleavage can reduce the amount of available PrP^C for conversion to PrP^{Sc} (Parkin *et al*., 2004).

Biosynthesis of PrP^C in cultured cells indicates that PrP^C undergoes cleavage as part of its normal metabolism (Mange *et al*., 2004) and that the processing of PrP^C appears to take place as it cycles between the plasma membrane and endosomal compartment (Harris, 1999, Mange *et al*., 2004, Tveit *et al*., 2005). This endosomal compartment may be part of the pathway that is involved in the conversion of PrP^C to PrP^{Sc} (Caughey *et al*., 1991, Borchelt *et al*., 1992 Harris, 1999). Recently, it has been shown that the generation of the truncated fragment C1 occurs in a late compartment of the secretory pathway and is independent of lipid rafts (Walmsley *et al*., 2009) as blocking exit of proteins from the endoplasmic reticulum reduced the amount of C1 fragment produced.

The precise nature of how PrP^C is processed is not fully understood. ADAM10 and ADAM17 (TNF-alpha converting enzyme), members of the ADAM family of cell surface disintegrin and metalloprotease proteins are linked to the formation of truncated PrP fragments (Vincent *et al*., 2001) as ADAM 10 and ADAM17 inhibitors result in a reduction of the N1 fragment (Alfa Cisse *et al*., 2008). However a paper by Endres *et al*., (2009) shows that ADAM 10 and TACE alone are not sufficient to

cleave PrP^C *in vitro* (Endres *et al*., 2009), this suggests that other factors must be involved in this cleavage process. ADAM 9 has been suggested as a candidate for alpha cleavage but requires ADAM 10 to activate its α-secretase activity (Cisse *et al*., 2005). Most recently ADAM 23 has been shown to interact and co-localise with PrP (Costa *et al*., 2009) but its role in cleavage is undefined.
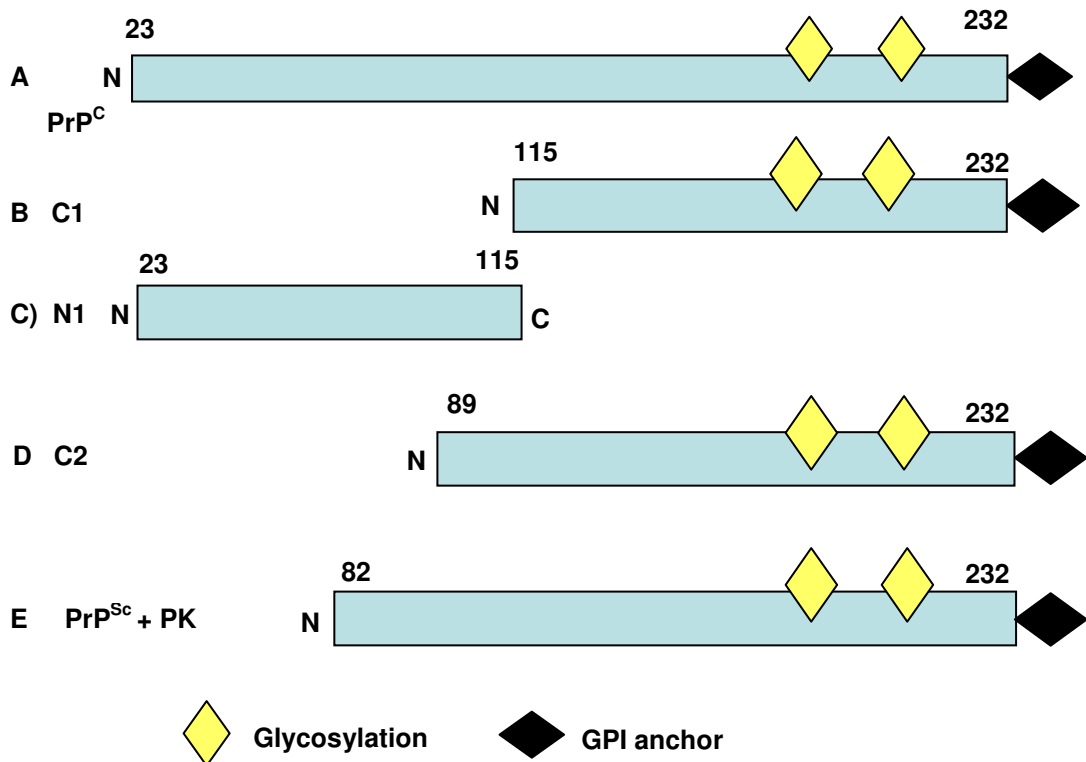
Figure 1.6 The different catabolites of PrP

A) PrP after the cleavage of the N- terminus, with the addition of two glycosylation sites and attached to the membrane by a GPI anchor. The molecular weight of this form of PrP is 33-35 kDa B) C1 catobolite that is produced after cleavage at amino acid 115 in relation to ovine PrP. This catobolite has a molecular weight of 18 kDa. C) The N1 catobolite that is produced after cleavage at amino acid 115. This catobolite has a molecular weight of 9 kDa. D) C2 catobolite that is produced after cleavage within the octapeptide region of PrP at approximately amino acid 89 after exposure to ROS. The molecular weight of the C2 catobolite is 21 kDa. E) PrP^{Sc} after digestion with PK. This form of PrP has a molecular weight of 26-29 kDa.

Catabolites of PrP may have functional importance within the brain as the C1 fragment has been shown to be pro-apoptotic (Sunyach *et al*., 2007) which is contradictory to the function of the N1 fragment which has been shown to down regulate p53 transcription and protects cells against caspase-3 activation (Guillot-Sestier *et al*., 2009). This suggests that under normal circumstances the N1 fragment is likely to be dominant and may represent the neuroprotection function associated with PrP^C. It can be hypothesised that under disease situations the C1 fragment becomes dominant and activates pro-apoptotic stimuli that lead to neuronal death within the brain. An alternative hypothesis would be that alpha cleavage results in loss of neuroprotective function as full-length PrP^C itself is reduced. At the moment these theories are yet to be investigated.

PrP can also be alternatively cleaved within the octapeptide region (β- cleavage) to generate a 21kDa C-terminal fragment (C2) and corresponding N-terminal fragment (N2) (Caughey *et al*., 1989, Chen *et al*., 1995, Parchi *et al*., 1999, Mange *et al*., 2004 Watt *et al*., 2005). *In vitro* β-cleavage takes place after exposure to reactive oxygen species (ROS) in the presence of a copper ion (McMahon *et al*., 2001, Watt *et al*., 2005). The C2 and N2 fragments do not appear to have any effect on the p53 pathway and show no pro-apoptotic or anti-apoptotic functions which have been associated with C1 and N1. It is possible that C2 and N2 could be potential neurotoxic intermediates in prion conversion as these fragments are not commonly observed under normal circumstances.

Similar size fragments have also been generated by the action of calpains (Yadavalli *et al*., 2004, Watt *et al*., 2005). As well as C1, C2 and corresponding N1 and N2 different sized fragments have been found in human brain (Gatti *et al*., 2002, Pan *et al*., 2002). Similar small fragmentss have been detected in ovine brain, epididymal fluid and milk (Pan *et al*., 2002, Maddison *et al*., 2007, Didier *et al*., 2008). The functions of these additional catabolites have not be investigated and it is unknown if they are involved in disease processes.

It is important to gain an understanding of PrP shedding and cleavage from the cell surface as increased shedding of the prion protein may reduce the amount of protein available for the conversion of PrP^C to PrP^{Sc} (Parkin *et al*., 2004, Walmsley *et al*., 2009). The hydrophobic region has been shown to be neurotoxic *in vitro* and *in vivo* (Bergstrom *et al*., 2005, Fioriti *et al*., 2005) and is dependent on the presence of the palindrome sequence which is located at amino acid 116-123 in ovine PrP (Figure 1.4) (Jobling *et al*., 1999). Without the palindrome sequence PrP^C may not be converted into PrP^{Sc} (Norstrom & Mastrianni, 2005). If the central region of PrP is indeed essential for conversion then α cleavage of PrP could be an important mechanism in disrupting this process.

The proteolytic processing of PrP^C and the formation of C1 has been investigated previously *in vivo* and *in vitro*. These studies were carried out on ovine PrP^C (Kuczius *et al*., 2007a) bovine PrP^C (Kuczius *et al*., 2007a) human PrP^C (Chen *et al*., 1995, Jimenez-Huete *et al*., 1998, Laffont-Proust *et al*., 2006, Kuczius *et al*., 2007a, Kuczius *et al*., 2007b) porcine PrP^C (Nieznanski *et al*., 2005) and murine PrP^C (Laffont-Proust *et al.*, 2005, Kuczius *et al*., 2007a).

In some of these studies the percentage of C1 observed was highly variabale between individual animals of the same species (Jimenez-Huete *et al*., 1998, Laffont-Proust *et al*., 2005). The percentage of C1 was also reported to vary between different species, for example Laffont-Proust *et al*., (2006) reported that the percentage of C1 differed between primates and rodents with higher signal intensities of C1 in primates. In a seperate study by Kuczius *et al*., (2007a) the signal intensity of C1 was higher than full-length PrP in sheep, human and cattle but mice showed dominance for full-length PrP. The presence of α-cleavage products has not yet been demonstrated for peripheral tissue, possibly because the total level of PrP is considerably lower than in brain.

Overall these studies show that C1 is produced in substantial amounts in the brains of many species. However, the function of this proteolytic event and the role this has on TSE susceptibility is still unknown.
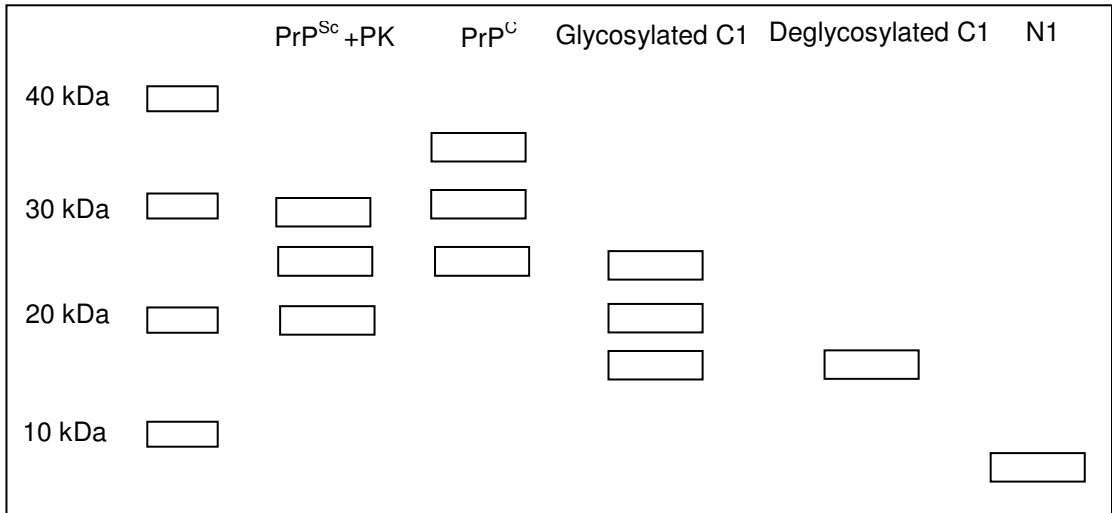
Figure 1.7 Different PrP catabolites and their apparent molecular weight in SDS PAGE
Approximation of molecular weight for the different fragmentss of PrP. PrP^{Sc} with PK the di-glycosylated protein is detectable at 26-29 kDa, mono-glycosylated protein at 23-25 kDa and unglycosylated protein at 20-22 kDa. PrP^{C} the di-glycosylated protein is detectable at 33-35kDa, mono- glycosylated protein at 26-29 kDa and unglycosylated protein at 23-25 kDa. The C1 fragment is glycosylated; diglycosylated C1 migrates at 23-25 kDa, monoglycosylated C1 at 20-22 kDa and unglycosylated C1 at 18 kDa. The corresponding N1 fragment is detectable at 9 kDa.

1.5 Aim

To investigate the role of alpha cleavage in scrapie disease susceptibility.

1.5.1 Hypotheses

a) The total amount of full-length PrP^C in sheep brain is associated with differences in scrapie susceptibility and incubation periods.

b) The total amount of the C1 fragment in sheep brain is associated with differences in scrapie susceptibility and incubation periods.

c) The ratio between full-length PrP^C and C1 is important in scrapie disease susceptibility or resistance.

1.5.3 Main Objective

1) Is the amount of C1 associated with polymorphisms located at codons 136, 154 and 171?

C1 as a percentage of total PrP^C was measured in the brains of healthy sheep with scrapie susceptible and resistant *PRNP* genotypes at codons 136, 154 and 171.

1.5.4 Subsidiary objectives

1) Is the degree of alpha cleavage affected by brain area/ and or age?

As PrP^{Sc} accumulates at different levels in different brain areas it was investigated whether the degree of alpha cleavage is affected by brain area and/or age. C1 as a percentage of total PrP^C was measured in four different brain areas. The amount of C1 in animals of a few days of age and in animals of up to 10 years of age was also compared.

2) How accurately do PrP transgenic mouse models reflect the level of alpha cleavage observed in sheep brain?

PrP transgenic mice are often used as alternative TSE models to replace experimental challenges of ruminants, but it remains to be shown that PrP is processed in these mice similarly to sheep. To investigate whether and to what level PrP is cleaved in PrP transgenic mice, 10 different mouse lines were analysed. (Nine transgenic and one control non-transgenic). These transgenic mice expressed murine, ovine or bovine PrP at different expression levels.

3) Does alpha cleavage occur at significant levels in peripheral ovine tissues to affect susceptibility to scrapie?

Animals are most likely exposed to the infectious prion agent through the peripheral route which implies that processing of PrP in peripheral tissue can have a significant effect on susceptibility, but it remains to be established if alpha cleavage is active in the periphery. Methods were examined to analyse the low levels of PrP in sheep lymph nodes and spleen.

Chapter 2 Material and methods

All chemical are from BDH unless otherwise stated. All experiments were carried out at room temperature unless otherwise stated.

2.1 Methods for protein analysis

2.1.1 Preparation of sheep and mouse brain homogenate

100 mg of brain tissue was homogenized in 1 ml of lysis buffer (50 ml of Phosphate buffered saline (PBS) pH 7.5 [Oxoid] in 100 mls of dH_2O, containing 0.25 g sodium deoxycholate (0.5%) and 250 μl of (5%) non ident (NP40) [Sigma]) to give a 10% (w/v) homogenate. Samples were centrifuged at 1000 x g for 10 minutes at 4°C. Supernatants were frozen in liquid N_2 (LN_2) and stored at -70°C. 10 μl of 100 mM phenylmethylsulfonyl fluoride (PMSF) [Sigma] and 100 mM N-ethylmaleimide (NEM) [Sigma] were added to prevent the generation of degradation products.

2.1.2 Sucrose gradient of brain homogenate

100 mg of brain tissue was homogenized in 1 ml of buffer A (10.95 g sucrose in 50 ml dH_2O containing 10 ml 10 mM $MgCl_2$, 5 ml 20 mM $NaHCO_3$ and 2.5 ml 20 mM KCl) pH 7.5. 57.5 μl of 100 mM PMSF [Sigma] and 12 μl of 100 mM Dithiothreitol (DTT) were added. Samples were centrifuged at 3000 x g for 10 minutes at 4°C. The pellet was then resuspended in 0.8 ml of buffer A and centrifuged at 3000 x g for 10 minutes at 4°C. Supernatants were overlayed onto a 0.4 ml 0.85 M sucrose gradient. Samples were centrifuged at 100,000 x g for 1 hour at 4°C. Supernatants were frozen in LN_2 and stored at -70°C.

Sucrose gradients are used to separate particles of different densities. In the above case a density barrier was used where the sample was overlayed on top of the sucrose. Therefore, the heavier more dense macromolecules migrated to the bottom of the tube faster than the lighter material. The lighter material stayed at the interface of the sucrose bed and the heavy material was pelleted at the bottom of the tube.

What components sediment out or stay at the interface will depend on the concentration of the sucrose bed.

2.1.3 Total protein estimation – the bicinchoninic acid (BCA) assay

Total protein estimation was carried out following the microwell plate protocol of a BCA protein assay kit [Pierce]. The BCA assay detects cuprous ions generated from cupric ions by reacting with protein in the samples under alkaline conditions. To a 3 μl aliquot of brain homogenate 57 μl of lysis buffer (PBS [Oxoid], containing 0.5% sodium deoxycholate and 0.5% of NP40) was added, i.e. 1:20 dilution of original homogenate. A range BSA standards [Pierce] were prepared following the BCA protocol (2.1.3 and Table 2.1) using the lysis buffer as a diluent. 25 μl of each standard and diluted sample were added in duplicate to wells of a 96 well microplate. The BCA working reagent was prepared by combining BCA reagents A and B [Pierce] in a ratio 50:1. 200 μl of this working reagent was added to the standards and diluted samples. The working reagent was also used as the negative control blank. The microplate was incubated at 37°C for 30 minutes and then cooled to room temperature (RT). The samples and standards were read and analysed on a molecular devices v-max microplate reader. Absorbance was read at 570 nm and from the standards a sample curve was produced. Protein concentrations for each sample were derived from the Bovine serum albumin (BSA) standard curve.

Table 2.1 Dilution scheme for standard test tube protocol and microplate procedure

Vial	Volume of diluent	Volume of BSA	Final BSA concentration
A	125 μl	375 μl stock	1500 μg/ml
B	325 μl	325 μl stock	1000 μg/ml
C	175 μl	175 μl of vial A	750 μg/ml
D	325 μl	325 μl stock of vial B	500 μg/ml
E	325 μl	325 μl stock of vial D	250 μg/ml
F	325 μl	325 μl stock of vial E	125 μg/ml
G	100 μl	150 μl stock of vial F	50 μg/ml

2.1.4 Protein deglycosylation

25 µl of 10% (w/v) sheep brain homogenate was denatured in 1 x glycoprotein denaturing buffer [0.5%) sodium dodecylsulphate (SDS), (1%) β-mercaptoethanol] by boiling at 100°C for 10 minutes. Samples were incubated with 1000 U/ml of peptide N-glycosidase F (PNGase F) [New England Biolabs] in 50 mM $NaPO_4$, pH 7.5 (1 x G7 reaction buffer, New England Biolabs) with 1% NP40 [New England Biolabs] at 37°C for 2 hours, according to the manufacturer's instructions. 200 µl of methanol and 100 µl of chloroform were then added to the sample reaction before centrifugation at 2000 x g for 10 minutes. The reaction was stopped by freezing at -20°C or by SDS denaturation at 100°C. When samples were ready to be used for SDS gel electrophoresis (2.1.6) they were centrifuged at 13000 x g, supernatants were discarded and pellets resuspended in 25 µl of 4 x laemmli (LDS) sample buffer [Invitrogen].

2.1.5 Proteinase K digestion of brain homogenate

0.5 µl of Proteinase K was added from a stock solution of 5 mg/ml (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7) to 20 µl of a 10% (w/v) sheep brain homogenated prepared in NP-40 lysis buffer and incubated for 1 hour at 37°C.

2.1.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Principles of SDS Gel electrophoresis

Native polyacrylamide gel electrophoresis is used to separate individual proteins by size, shape and charge. PAGE can also be performed under denaturing conditions in the presence of Sodium Dodecyl Sulfate (SDS-PAGE) or under reducing and denaturing conditions in the presence of DTT or beta mercaptoethanol. Prior to SDS-PAGE the protein is denatured by heating the sample in the presence of a detergent containing SDS. SDS denatures secondary and tertiary structures and applies a negative charge to each protein in proportion to its mass. Protein samples fractionated by denaturing SDS-PAGE are therefore resolved according to their molecular weight regardless of charge.

When mixtures of proteins are loaded onto a gel and an electric current applied, smaller proteins migrate faster than larger proteins through the gel. The rate of movement is influence by the gel's pore size and the strength of the electric field. Pores in polyacrylamide gels are quite small therefore only small proteins and peptides are able to pass through.

Prior to electrophoresis brain tissue samples were deglycosylated (2.1.4) and incubated in 4 x LDS sample buffer (final concentration glycerol 10%, Tris 157 mM, lithium dodecyl sulphate 2%, Ethylenediaminetetracetic acid (EDTA) 0.51 mM, SERVA Blue$^®$ G250 0.22 mM, Phenol Red 0.175 mM pH 8.5, [Invitrogen] for 10 minutes at 100°C. Proteins were then separated on the basis of molecular weight by gel electrophoresis at 150V for 1 hour using Novex 12% Bis-Tris Gels 1.0 mm [Invitrogen] in NuPAGE$^®$ 1 x MES SDS running buffer (MES 50 mM pH 7.2, Tris 50 mM, SDS 0.1%, EDTA 1 mM, [Invitrogen]). Lysis buffer (2.1.1) and NuPAGE LDS 4 x sample buffer were used as negative controls. SeeBlue [Invitrogen] and magic-marker [Invitrogen] were used as molecular weight markers.

2.1.7 Western blot

Principles of the Western blot

Transfer of the proteins fractionated by SDS-PAGE to a solid support membrane can be achieved by electroblotting using semi dry or tank transfer systems. In this assay a sandwich of gel and a solid support membrane (nitrocellulose or polyvinylidine fluoride (PVDF) is compressed in a cassette and immersed in buffer between two parallel electrodes. A current is passed through the gel, which causes the separated proteins to electrophorese out of the gel and onto the solid support membrane PVDF. The efficiency with which proteins transfer to the blot is dependent on the protein binding capacity of the membrane used and the transfer method. While SDS is required to facilitate migration of the protein out of the gel in response to an electric current, SDS can also interfere with binding of the protein to the membrane itself.

Once the proteins have been transferred to the solid support membrane it is essential that the membrane is blocked prior to the addition of the primary antibody. A blocking agent such as BSA or dried milk powder in PBS, will inhibit remaining hydrophobic binding sites on the membrane and will consequently reduce background and prevent binding of the primary antibody to the membrane itself. After blocking the membrane, the primary antibody can be added for 1 hour at 37°C or overnight at 4°C with gentle agitation. The time of incubation will vary for different antibodies. Following incubation of the primary antibody, the blot is washed in wash buffer containing PBS with the addition of Tween 20 before addition of a secondary labelled antibody.

The method of detection is dependent upon the label that has been conjugated to the secondary antibody. The most common antibody label used in Western blotting is an enzyme such as alkaline phosphatase or horseradish peroxidise. Chemiluminescent substrates are employed which emit light upon conversion by the enzyme. The light emitted at the site of substrate conversion can therefore be captured on x-ray film.

2.1.7.1 Transfer of separated proteins to a membrane

Proteins separated by SDS gel electrophoresis were transferred to PVDF membrane [Millipore] at 25V for 1 hour, using the NuPAGE transfer system, in 1 x NuPAGE transfer buffer [Invitrogen], final concentration, bicine 25 mM, Bis-Tris (free base) 25 mM, EDTA 1.0 mM, chlorobutanol 0.05 mM) and 20% methanol [Fischer].

2.1.7.2 Detection of protein bound to a membrane

To reduce unspecific binding of antibodies, membranes were blocked with 1% blocking solution [Roche Diagnostics] for 1 hour. Overnight incubation of the primary antibody was carried out in 0.5% blocking solution [Roche Diagnostics] at RT. Membranes were then washed 2 x 10 minutes with TBS and 1% Tween (TBST) and 2 x 10 minutes in 0.5% blocking solution [Roche Diagnostics]. Incubation of HRP-conjugated anti-mouse secondary antibody (0.08 μg/ml) [Stratech] was carried out in 0.5% blocking solution [Roche Diagnostics] for 1 hour. Membranes were washed in TBST to remove excess secondary antibody prior to incubation with

chemiluminescent substrate [Roche Diagnostics] for 1 minute followed by exposure to Kodak XAR-5 film [Roche Diagnostics]. The film was developed for 1 minute, 3 minutes and 10 minutes, respectively.

2.1.7.3 Stripping of PVDF membrane

To allow for re-probing of the PVDF membrane with a second antibody, blots were stripped with "restore stripping" solution [Thermo Scientific] by shaking overnight. Prior to reprobing blots were washed in TBS, TBST and 0.5% blocking solution [Roche Diagnostics] following instructions for (2.1.7.2).

2.1.8 Coomassie stain of PVDF membrane

To visually observe membrane bound proteins on a NuPAGE gel a Coomassie blue stain was performed. The gel was incubated for 1-2 hours in Coomassie brilliant blue stain (0.625 g coomassie blue, 125 mls methanol [Fischer], 23 mls acetic acid and 102 mls of dH_2O) either before or after transfer to the PVDF membrane. The gel was then incubated for 1-2 hours with destain solution (23% Ethanol, 7% acetic acid) with approximately 6 washes or left overnight if necessary.

2.1.9 Testing Protein degradation kinetics

300 mg of brain tissue was thawed and left in the fridge for 2, 24 and 48 hours without the presence of protease inhibitors. At the indicated time points 100 mg of the tissue was prepared as described in (2.1.1), sheep brain homogenate protocol. The sample was then deglycoslyated following the protein deglycosylation protocol (2.1.4). The sample was stored at -20°C ready for polyacrylamide gel electrophoresis (2.1.6).

500 mg of brain tissue was thawed for 15 minutes. The sample was then refrozen at -20°C for 1 hour. This process was repeated four times. 100 mg of tissue was then prepared following the sheep brain homogenate protocol (2.1.1). The sample was then deglycosylated following the protein deglycosylation protocol (2.1.4) and then stored at -20°C until polyacrylamide gel electrophoresis (2.1.6).

2.1.10 Immunoprecipitation

Tissue homogenates (10% w/v) were prepared in extraction buffer (0.5% NP-40, 0.5% sodium deoxycholate, PBS [Oxoid]), clarified by centrifugation (913 x g, 10 minutes) and the supernatants collected. Aliquots (10 µl) of tissue homogenate were mixed with 1 µl of IgM MAb or 1 µl of a non PrP related control IgM [Sigma] in 100 µl final volumes in PBS [Oxoid], and incubated on a rotary mixer overnight at 4°C. Rat anti-IgM conjugated dynabeads (10 µl) [Invitrogen] were added to each sample and mixed on a rotary mixer for 60 minutes. Samples were placed in a magnetic rack to pull down the beads and the attached proteins. The beads were washed three times in PBS supplemented with 0.1% Tween, resuspended in 25 µl 1 x NuPAGE LDS sample buffer (Invitrogen) and sonicated 3 x 30 seconds in a water bath. Each sample was then boiled for 10 minutes at 100°C. Electrophoresis and Western blotting were then performed as previously described (2.1.6-2.1.7.2).

2.1.11 Analysis of the amount of PrP in ovine brain tissue homogenate by dissociation enhanced lanthanide fluoroimmunoassay DELFIA®

2.1.11.1 Preparation of brain homogenate for DELFIA®

PrP^C was extracted from total protein brain homogenate (100 mg/ml) NP-40 lysis buffer, 1 M PMSF [Sigma] and 1 M NEM [Sigma] by mechanical homogenisation in 2 M Gnd HCl [Sigma] and (0.5%) Triton X-100 [Sigma]. Brain homogenate was then diluted in DELFIA® assay buffer [Perkin Elmer Life Sciences] leading to a final concentration equivalent to 10 mg/ml of original brain tissue. Proteins insoluble in 2 M Gnd HCl (PrP^{Sc}) were separated from those that were soluble (PrP^C) by centrifugation at 12000 x g for 10 minutes. Pellets were resuspended in 6 M Gnd HCl prior to dilution in DELFIA® assay buffer [Perkin Elmer Life Sciences] to a concentration equivalent to 10 mg/ml of original brain tissue.

2.1.11.2 Preparation of recombinant PrP standard for DELFIA®

Sheep recombinant PrP (Kind gift from Dr. Louise Kirby, Compton) was serially diluted 2 fold in DELFIA® assay buffer [Perkin Elmer Life Sciences] (100 – 0.3905 ng/ml) for use as a standard. All samples that were used in the DELFIA® assay were within this range.

2.1.11.3 Spectrophotomeric determination of recombinant PrP concentration

Concentration of total protein in recombinant PrP was determined using spectrophotomeric analysis. In 1.5 ml eppendorfs, a 20 μl aliquot of the recombinant protein was added to 200 μl of dH$_2$O (to give 1:10 dilution), and a 2 μl aliquot of recombinant protein was added to 200 μl of dH$_2$O (to give 1:100 dilution). Samples were mixed well and transferred to a silica spectrophotometer cuvette [Sigma]. Absorbance at 280 nm and 320 nm was determined using a UV spectrophotometer.

The following calculation was performed to determine the total protein concentration (mg/ml). The extinction Co-efficient for PrP is 57780.

Absorbance (280-320) x Molecular Weight

    Extinction Co-efficient (57780)   **=   protein concentration (mg/ml**)

2.1.11.4 DELFIA® time-resolved fluorescence immunoassay

Capture antibody FH11 (1 mg/ml) was bound to 96 well plates by overnight incubation at 4°C. Plates were then blocked with 2% (BSA) [Roche Diagnostics] in sterile 1 x PBS [Oxoid] with 3 M NaN$_3$ (sodium-azide) for 1 hour with shaking. The block solution was discarded and the plate was incubated with samples and standards, shaking at for 1 hour. Samples and standards were discarded and the plate was incubated with europium (Eu^{3+}) labelled detector antibody 8H4 (kind gift from Mr Declan King, Neuropathogenesis Division, Roslin Institute) shaking for 1 hour. The plate was then incubated with DELFIA® enhancement solution [Perkin Elmer Life Sciences]. After shaking for 5 minutes Eu^{3+} emission was read (615 nm), using a time-resolved technique. Between each step, the plate was washed in 1 x DELFIA® wash concentrate [Perkin Elmer Life Sciences] using a DELFIA® automatic plate washer [Wallac]. The Wallac 1420 workstation [Wallac] was used to analyse absorbance from the standards and samples and to produce the standard curve.

2.2 General methods for DNA analysis

2.2.1 DNA extraction from sheep brain tissue

DNA was extracted from sheep brain tissue using the following method. To a thin slice of tissue, 200 μl of PBS [Oxoid], 200 μl of tissue lysis buffer [Applied Biosystems] and 200 μl of Proteinase K [Qiagen] was added and incubated overnight at 37°C. After overnight incubation 400 μl of phenol and 400 μl of chloroform was added and the sample centrifuged at 1200 x g for 3 minutes (This step was repeated twice). The aqueous phase was transferred to a fresh eppendorf in each case. 400 μl of chloroform was added and the aqueous phase was transferred to a fresh eppendorf. To the aqueous phase 15 μl of 3M NaAc pH 5.2 and 225 μl of cold 100% ethanol [Fischer] was added. Samples were stored at -20°C for 30 minutes. The sample was then centrifuged at 1200 x g for 10 minutes and the supernatant discarded. 500 μl of cold 75% ethanol [Fischer] was added to sample and the sample was centrifuged at 1200 x g for 5 minutes. The supernatant was discarded and the pellet was left to air dry at RT. The pellet was re-suspended in 100 μl of dH$_2$O and stored at -20°C.

2.2.2 Spectrophotomeric determination of DNA sample concentration and purity

DNA sample concentration was determined by spectrophotomeric analysis. Absorbance at 260 nm and 280 nm was determined using UV spectrophotometer (Beckman DU$^{®}$ 650). DNA Samples were diluted 1:40 prior to spectrophotometric analysis. DNA purity was determined by dividing the absorbance value at 260 nm by the value at 280 nm.

2.2.3 Agarose gel electrophoresis

DNA was separated by electrophoresis in a 1% agarose gel [Roche Diagnostics] with 0.5µg/ml ethidium bromide [Sigma] for 1 hour at 120 V. A 1 Kb DNA ladder (12216 – 75 base pair fragments); [New England Biolabs] was used as a size standard. A positive control and a negative control were loaded alongside experimental samples. The resulting DNA bands were visualised using UV light and photographed using video imager [Appligene].

2.2.4 PCR of genomic sheep DNA

The target region of the prion gene was amplified using the polymerase chain reaction (PCR)

Sample mix per reaction:

dH$_2$O - 38.7 µl

X 10 reaction buffer [Applied biosystems] – 5 µl

dNTPs [Promega] - 0.4 µl

Oligo MPS-52d [Sigma] $^{5'}$ CTTACGTGGGCATTTGATGC $^{3'}$ - 0.2 µl

Oligo MPS+16u [Sigma] $^{5'}$ AACAGGAAGGTTGCCCCTATCC $^{3'}$ - 0.2 µl

Taq polymerase [Invitrogen] – 2.5 µl

DNA – 3 µl

PCR conditions:

95°C 3 minutes: 1 cycle

95°C 30 seconds: 40 cycles

63°C 30 seconds: 40 cycles

72°C 1 Minute: 40 cycles

72°C 10 Minutes: 1 cycle

Held at 4°C

PCR conditions vary depending on the oligonucleotides that are being used. For each reaction a positive control (a sample known to contain amplifiable DNA) and a negative sample (dH_2O) were added.

2.2.5 Detection of the G1, G2, G3 mutant and wild type *prnp* alleles by PCR

The presence of the G1 mutant allele was determined by mismatch PCR using an oligo specific for the G1 mutation. These mice express an alteration at codon 180 (N180T), which disrupts N-linked glycosylation at this site.

G1 mutant sample mix per reaction (50 μl)

H_2O - 40.1 μl

PCR reaction buffer x 10 [Invitrogen] – 5 μl

10mM dNTPs [Promega] – 1 μl

50mM $MgCl_2$ [Invitrogen] - 1.5 μl

Taq polymerase [Invitrogen] – 0.4 μl

G1 oligo mix - 1μl of a 3 oligo mix [9910 (100 pmol/μl), 9911 (100 pmol/μl), 9912 (100 pmol/μl)]

Oligo9910   [5] AACCTCAAGCATGTGGCAGGGGCTGCGGCAGCTGG [3]

Oligo 9911   [5] GCTGCTTGATGGTGATAG [3]

Oligo 9912   [5] TCAGTGCCAGGGGTATTAGCCTATGGGGGACACAG [3]

DNA – 1 μl

G1 mutant PCR conditions:

94°C 3 minutes: 1 cycle

94°C 50 seconds: 35 cycles

60°C 30 seconds: 35 cycles

72°C 1 minute: 35 cycles

72°C 10 minutes: 1 cycle

Held at 4°C

The presence of the G2 mutant allele was determined by mismatch PCR using an oligo specific for the G2 mutation. These mice express an alteration in codon 196 (N196T), which disrupts N-linked glycosylation at these sites. The presence of the G3 mutant allele was also determined by mismatch PCR using an oligo specific for the G1 or G2 mutant allele.

G2 mutant sample mix per reaction (50 μl)

H_2O - 40.1 μl

PCR reaction buffer x 10 [Invitrogen] – 5 μl

10mM dNTPs [Promega] – 1 μl

50mM $MgCl_2$ [Invitrogen] - 1.5 μl

Taq polymerase [Invitrogen] – 0.4 μl

G2 oligo mix – 1 μl of a 3 oligo mix [9910 (100 pmol/μl), 9912 (100 pmol/μl), 9913 (100 pmol/μl)

Oligo 9910 [5] AACCTCAAGCATGTGGCAGGGGCTGCGGCAGCTGG [3]

Oligo 9912 [5] TCAGTGCCAGGGGTATTAGCCTATGGGGGACACAG [3]

Oligo 9913 [5] CATCGGTCTCGGTGAAGG [3]

DNA – 1 μl

G2 mutant PCR conditions:

94°C 3 minutes: 1 cycle

94°C 50 seconds: 30 cycles

60°C 30 seconds: 30 cycles

72°C 1 minute: 30 cycles

72°C 10 minutes: 1 cycle

Held at 4°C

The presence of the glycosylation wild-type allele for G1, G2 and G3 mice was determined by Mismatch PCR. PCR conditions were the same as for the G1 mutant allele.

Glycosylation wild type sample mix per reaction (50 μl)

H_2O - 40.1 μl

PCR reaction buffer x 10 [Invitrogen] – 5 μl

10mM dNTPs [Promega] – 1 μl

50mM $MgCl_2$ [Invitrogen] - 1.5 μl

Taq polymerase [Invitrogen] – 0.4 μl

Oligos – 1 μl

Oligo 9910 [5] AACCTCAAGCATGTGGCAGGGGCTGCGGCAGCTGG [3]

Oligo 9912 [5] TCAGTGCCAGGGGTATTAGCCTATGGGGGACACAG [3]

G1-WT [5] GCTGCTTGATGGTGATAT [3] [9910 (100 pmol/μl), G1-WT (100 pmol/μl), 9912 (100 pmol/μl)

G2-WT [5] CATCGGTCTCGGTGAAGT [3] [9910 (100 pmol/μl), G2-WT (100 pmol/μl), 9912 (100 pmol/μl)

DNA – 1 μl

2.2.6 Activated charcoal purification of PCR products

0.8 g of activated charcoal [Sigma] was added to 5 ml of dH_2O to make a PCR clean up mix. 15 μl of the clean up mix was added to 40 μl of the PCR reaction which was mixed gently and incubated at RT for 10 minutes. The PCR reaction was centrifuged at 12000 x g for 5 minutes to pellet the charcoal. 8 μl of this cleaned up-amplified product was used in the sequencing reaction.

2.2.7 Sequence reaction

Make up master mix as below.

Sample mix per reaction:

H_2O – 12 μl

Big dye kit 3.1 [Applied biosystems] – 2 μl

X 5 buffer [Microzone limited] – 3 μl

Oligo 9612 [Sigma] $^{5'}$ GGT GAA GTT CTC CCC CTT GGT $^{3'}$ – 1 μl

Oligo MPS 325 [Sigma] $^{5'}$ GCCACATGGTGGTGGAGGC $^{3'}$ – 1 μl

Purified PCR product – 2 μl

To a strip tube 2 μl of purified PCR product and 18 μl of sample reaction mix were added. The sequence reaction was carried out as below.

PCR Sequence reaction conditions:

96°C for 10 seconds – 25 cycles

53°C for 15 seconds – 25 cycles

60°C for 4 minutes – 25 cycles

Held at 25°C

To each sample 5 μl of 125 mM EDTA and 60 μl of 100% ethanol [Fischer] were added. Samples were mixed and left for 15 minutes. Samples were centrifuged at 1400 x g for 30 minutes at 4°C and then centrifuged at 1000 x g at 4°C. To each sample 60 μl of 70% ethanol [Fischer] were added and lightly mixed. Samples were centrifuged at 1400 x g for 15 minutes at 4°C. Excess alcohol was removed and the samples centrifuged at 1000 x g at 4°C. Samples were left to air dry for approximately 30 minutes. 12 μl of Hi-di formamide [Applied Biosystems] were added to each sample and lightly mixed. The samples were then centrifuged at 1000 x g at 4°C. 12 μl of each sample were loaded onto the Applied Biosystems 3130 genetic analyser. Data was viewed and analysed using the Applied Biosystems sequencing analysis software version 5.2 and the software chromas lite V2.01 [Technelysium Pty Ltd].

2.3 Methods for monoclonal antibody production

2.3.1 Preparation of R10 media

To make R10 cell growth medium, 50 ml of foetal calf serum (FCS) and 15 ml of AB/AM (5 ml antimycotic solution, 5 mls L-glutamine and 5mls of sodium pyruvate) was added to 500 ml of Rosewell Park Memorial Institute medium (RPMI) [Sigma]. When a new batch of medium was prepared, 2 ml of R10 medium was incubated at $37°C$ in 5% CO_2 for 24 hours to check for bacterial sterility. The remaining R10 media was stored at $4°C$.

2.3.2 Retrieval of cells from LN_2 storage

A vial of the required cell line was removed from LN_2 storage and defrosted in a $37°C$ water bath for 5 minutes. Once the cells were defrosted they were immediately transferred to 10 ml of fresh R10 media. The solution was mixed briefly and centrifuged at 1000 x g for 5 minutes. The supernatant was decanted into a fresh 50 ml centrifuge tube and stored at $-40°C$ (2.3.6) and to the remaining pellet 5 ml of fresh warm R10 media was added. The pellet was gently resuspended in the media and transferred to a $25cm^2$ cell culture flask. Cell density was checked under the microscope and more R10 was added if the cells were covering more than 90% of the flask. The $25cm^2$ flask was placed in a 5% CO_2 humidified incubator at $37°C$ and after several hours, cell density was checked under microscope. More R10 media was added if it was required.

2.3.3 Single cell cloning by limiting dilution

All reagents were warmed in a water bath, to $37°C$ before starting. Cells were detached from the surface of the well by pipetting the media thereby dislodging the cells. 10 µl of cell suspension was mixed with 10 µl of trypan blue. 10 µl of this 1:1 mix was pipetted under the coverslip of a haemocytometer and the number of cells in one of the large squares counted. The volume required to give 60 cells was calculated using the following equation: No. of cells x 10^4/ ml. 100 µl/well of the diluted cell suspension was added to the inner 60 wells of a 96 well plate. The 96 well plate was then incubated in a $37°C$ humidified incubator with 5% CO_2 for 7 days. After this

time the colony growth was recorded and the cells were fed with 100 µl of 20% FCS growth media and left to grow.

2.3.4 Culturing of cells expressing IgM monoclonal antibodies H1.8, H3.2 and H3.3

Cells were grown in R10 media RPMI (2.3.1) supplemented with 0.5 ml of mixed thymocyte media (MTM). Cells were incubated at 37°C with 5% CO_2 in $225cm^2$ flasks until confluency was reached. Cultured cells were fed approximately every 2 days with fresh R10 media. Hybridoma cells were prepared for passage by diluting approximately 1:10 with fresh R10 media.

2.3.5 Freezing down cell lines

To preserve a cell line, cells which had grown in $75cm^2$ flasks were dislodged from the surface and centrifuged at 1000 x g for 5 minutes. Media supernatant was decanted into a fresh 50 ml centrifuge tube and stored at -40°C for screening and antibody purification. The remaining pellet was resuspended in 5 ml of freezing medium [90% FCS, 10% dimethyl sulfoxide (DMSO)]. 1 ml of the cell/freezing medium solution was transferred to an individual vial. Vials were stored in a Nalgene® Cryo°C freezing container, filled with 250 mls of 100% isopropanol and stored at -80°C.

2.3.6 Collection and Purification of monoclonal antibody rich media

At any point during the expansion or freezing of the cell lines where media was taken off it was retained as antibody rich media and stored at -40°C.

500ml of saturated ammonium sulphate solution was added to 500 ml of cell free culture supernatant and stirred for 60 minutes. The solution was centrifuged at 3300 x g for 20 minutes. The supernatant was retained and the pellet resuspended in 30 ml of PBS [Oxoid]. The solution was concentrated down to a 1 ml final volume using a Biomax 50K NMWL 15 ml volume centrifugal filter device [Millipore]. The solution was centrifuged at 2000 x g until the desired 1ml final volume was achieved. 9 ml of PBS [Oxoid] was added to the centrifugal filer device and centrifuged to a 1 ml final

volume; this step was repeated two more times. Maltose (10% [w/v]) and sodium azide (0.001% [w/v]) were added to inhibit bacterial growth; the monoclonal antibodies (MAbs) were dispensed into 100 μl aliquots and stored at -40°C.

2.3.7 Isotyping of monoclonal antibodies

Isotyping of MAbs was carried out using a mouse monoclonal antibody isotyping kit [Roche]. Following the manufacturer's instructions 15 μl of antibody containing media was diluted 1:10 with 10% FCS growth media (150 μl final volume). This solution was added to the supplied development tube, which was agitated to resuspend the colour latex beads. The isotyping strip was the placed into solution. After approximately 5 minutes a blue band appeared on the strip to indicate the isotype of the MAb (Figure 2.1).
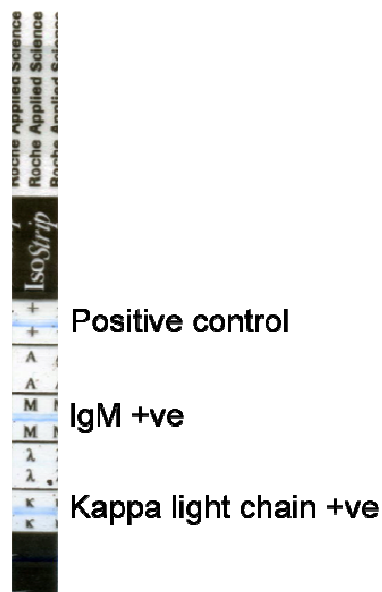
Figure 2.1 Isotype result for H3.2

Blue line indicates a positive result. When the control (+) turns blue this indicates that the test is working properly. This result confirms that H3.2 is an IgM antibody with a Kappa light chain.

2.3.8 Determinatation of MAb concentration by ELISA

<u>Principles of the ELISA technique</u>

The ELISA assay is used to determine the antigen concentration in unknown samples. To perform this assay one antibody (the capture antibody) is bound to the bottom of a plate well. The wells are then washed to remove any unbound antibody and blocked using an unspecific protein or protein mixture to prevent non-specific binding of the secondary antibody (the detector antibody). If the wells are not blocked then the secondary antibody may stick to non-specific proteins due to their charge. Common blocking agents used in the ELSIA assay include dried milk powder, bovine serum albumin and casein.

After the blocking step antigen is added and allowed to form a complex with the bound antibody. Unbound products are then removed with a wash step and a labelled second antibody (the detector antibody) is allowed to bind to the antigen. The second antibody is modified to carry a reporter enzyme, such as horseradish peroxidase or alkaline phosphatase which is designed to form a colour change when the enzyme reacts with its substrate. The sample is then rinsed to remove any unbound antibodies. If the antigen is present, a complex will have formed that includes the antibody bound to the well, the antigen and the enzyme conjugated antibody. The enzyme's substrate is then added. A colour change reveals the presence of enzyme-labelled antibody as well as its bound antigen. No colour change indicates that the antigen was not present. The assay is then quantified by measuring the amount of labelled antibody bound to the matrix, through the use of a colorimetric substrate.

2.3.8 Determination of MAb Concentration by ELISA

Wells of a 96-well immunlon 4 HXB microtitre plate [Sigma] were coated with anti-mouse IgM (μ-chain specific) capture antibody [Sigma], 100 ng/well in carbonate/bicarbonate coating buffer (0.318 g/L Na_2CO_3, 0.586 g/L $NaHCO_3$, pH 9.6) overnight at 4°C. Wells were washed four times with PBS and 0.1% (w/v) Tween 20 (PBST) and bocked with PBS and 5% FCS at 37°C for 1 hour. Well were then washed four times with PBST. Ten-fold serial dilutions of the MAb (1:1000 to 1:25600) and two-fold serial dilutions of the corresponding IgM control [Sigma], in

the range 200-0.78 ng/ml were prepared in dilution buffer. Aliquots (100 μl) of each MAb and standard serial dilution were added to triplicate wells of an appropriately coated microtire plate, incubated 60 minutes, 37°C, washed four times with PBST, and blot dried. The ELISA was then processed using goat anti-mouse IgM horseradish peroxidise (HRP) conjugated secondary antibody [Sigma]. The mean absorbance 450 nm for each standard serial dilution was calculated, corrected by subtracting the mean blank absorbance 450 nm for the non-specific binding of the HRP conjugated secondary antibody, and plotted against antibody concentration to produce a standard curve. Using the corrected mean absorbance 450 nm obtained for each MAb serial dilution, the MAb concentration was then determined from the standard curve and multiplied by the dilution factor to give the actual MAb concentration.

2.4 Computer based analyses

2.4.1 Statistical analysis

Measurement data are presented as mean ± standard deviation (S.D) or standard error of the mean (S.E). Any significant differences between samples in different groups were confirmed by REML (residual estimate maximum likelihood) analysis taking into account that various factors were unbalanced. REML analysis was carried out using the Genstat 10th edition software. When values of 100% were included in the analysis a non-parametric sign test was used for statistical analysis. Values of $P<0.05$ were accepted as significant.

2.4.2 Statistical power

Throughout this thesis it was difficult to collect a large number of samples. In some cases only two samples could be collected, this meant that the sample population was not large enough to carry out statistical analysis. If the sample size is large then the estimate of the population mean will be more precise and the statistical power will be high. If the sample size is small then the statistical power will be low. Throughout this thesis the sample size is relatively small which means that the statistical power is relatively low.

2.4.3 Semi Quantitative analysis of Western blots

An equal amount of brain homogenate (i.e. 5 µl) was also loaded and a loading control (alpha tubulin) was sometimes used to observe whether equal amounts of protein had been loaded. This was particularly important when comparing bands for different brain areas.

Western blot images were scanned using the Kodak MI image station. Using the Kodak MI software a region of interest was selected covering all parts of the film, the lanes and bands were automatically detected. Following band location and fitting, the Kodak MI software calculated the net intensity determined by the number and intensity of pixels within the band area. For each Western blot image three separate exposures were taken at 1, 3 and 10 minute intervals. In some cases direct exposure of the membrane onto the Kodak MI scanner was carried out, however as only some of the images were calculated using this method, only values calculated indirectly from the film have been included in this thesis.

In most cases the signal was saturated at the 10 minute exposure, this was indicated on the Kodak MI software when the peak of the over exposed band would become flat. This is indicated in Figure 2.2 where the peak for unglycosylated PrP is flatter compared to the peak for the C1 band. The Kodak MI software, in built program therefore gave an estimate of the maximum intensity. In cases where the signal was saturated the values were not included in the statistical analysis. By ignoring the bands that were saturated it is possible that the amount of full-length PrP or C1 calculated is an underestimate of the real value.

The net intensity was used for quantitative analysis of the bands. In cases of incomplete deglycosylation, these bands were included in the analysis along with any other additional fragments of PrP. A ratio between total PrP^C and C1 was therefore calculated. The molecular weight for each band was also calculated using the Kodak MI software using molecular weight markers as a standard.
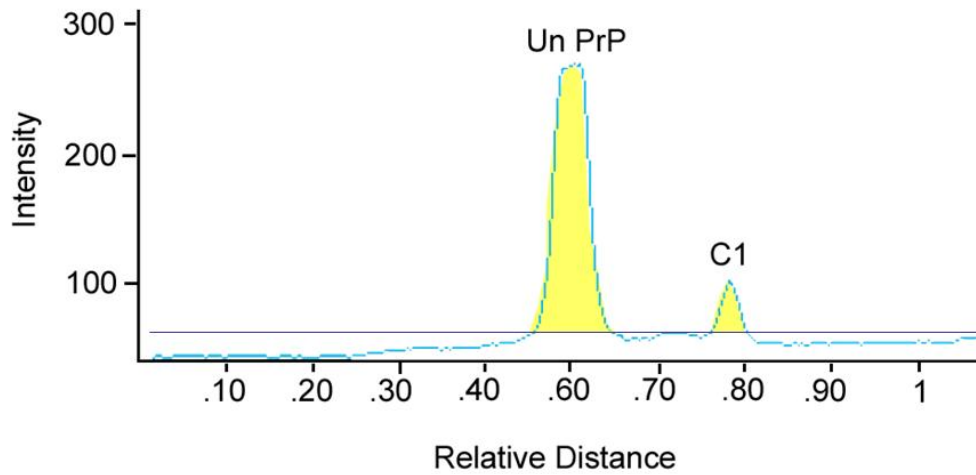
Figure 2.2 Densitometry profile of Western blot of unglycosylated PrP (Un PrP) and the C1 fragment from uninfected sheep

Example of PrP and C1 detected using anti-PrP monoclonal 6H4. Intensity of anti-PrP signal displayed on y-axis, relative distance from the top of gel on the x-axis. Area highlighted in yellow, was used for quantification of full-length PrP to C1 ratio. In cases where the signal was saturated, values were not included in the analysis.

2.4.4 Preparation of gel figures and nomenclature

Original Western blot images were transferred to Photoshop element 5.0 (Adobe, 2006) and greyscale mode applied. Images were cropped and labelled using the photoshop software. Where obvious gaps exist between different images these were taken from different gels and grouped together or in some cases they were non-continous gel images). No manipulation of the original images was carried out.

To explain the different sheep PrP genotypes, amino acid residue numbers refer to the ovine PrP sequence for example.

VLRQ is V136 L141 R154 Q171 and ALRR is A136 L141 R154 R171 with V = valine, L = leucine, R = arginine and Q = glutamine. In general amino acids are described in standard one-letter code. Ovine PrP alleles are also shown in three-codon standard nomenclature, e.g. VRQ is V136 R154 Q171. Genotypes in turn are shown as VRQ/VRQ, ARR/ARR etc.

2.5 Materials

2.5.1 Mice

Mice were bred and housed at the Neuropathogenesis Division, Roslin Institute, Edinburgh. All animal breeding and experimental work was carried out under Home Office legislation and approved by the local Ethics Committee. All transgenic animals have been approved by the Health and Safety Executive.

2.5.2 Sheep

Sheep were from the NPU flock, Compton flock, VLA and Moredun Research Institute.

Animals with the following genotypes were used:

ALRR/ALRR n= 6,

ALHQ/ALRR n= 5,

ALRQ/ALRR n= 12,

AFRQ/ALRR n= 1,

ALHQ/AFRQ n= 4,

ALHQ/ALRQ n=1,

VLRQ/VLRQ n = 10,

ALRQ/ALRQ n= 9,

VLRQ/ALRQ n= 4

VLRQ/AFRQ n=2.

Table I in appendix I provides additional information for each animal within each genotype group. Sequences were established between codon 86 and codon 202. Three animals had a known additional polymorphism in codon 112 with genotypes M112ARQ T112ARQ (n=2) and T112ARQ T112ARQ (n=1) (Appendix I).

2.5.3 Antibodies

The primary antibodies used in this study are shown in Table 2.2

Table 2.2 Primary antibodies used in Western blotting and for DELFIA assay

Antibody	Antigen	Type	Epitope	Source	Conc. (μg/ml)
BE12	PrP	Mouse monoclonal (IgG1)	23-90	J.Manster Institute Animal Health	0.1
FH11	PrP	Mouse monoclonal (IgG2b)	54-57	C.Birkett Institute Animal Health	1000
P4	PrP	Mouse Monoclonal (IgG)	93-99	r-biopharm	0.2
3F4	PrP	Mouse Monoclonal (IgG2a)	a.a109-112 human and hamster only	Signet Laboratories	0.5
BC6	PrP	Mouse Monoclonal (IgG)	a.a147-155	Sandra McCutcheon The University of Edinburgh	0.08
6H4	PrP	Mouse Monoclonal (IgG1)	a.a147-155	Prionics	0.1
H1.8	PrP	Mouse Monoconal (IgM)	a.a 147-166	Michael Jones The University of Edinburgh	1000
H3.2	PrP	Mouse Monoclonal (IgM)	a.a 157-176	Michael Jones The University of Edinburgh	1000
8H4	PrP	Mouse Monoclonal (IgG1)	a.a175-185	Man-Sun Sy, Case Western Reserve University, Cleveland USA	0.3
H3.3	PrP	Mouse Monoconal (IgM)	a.a 187-206	Michael Jones The University of Edinburgh	1000
ab-2	α-tubulin	Rat Monoclonal		Fischer Scientific	0.1

2.5.4 Cell lines

Cells were grown from individual cloning well plates or from vials from LN_2 storage to $75cm^2$ flasks and frozen down to preserve the cell line for future characterisation studies (2.3.5). Various batches of cells from the same cell line were frozen on different days (Table 2.3). This was carried out so there was a back up in case something went wrong with the freezing process on a particular day. This was not the case for H3.2 which unfortunately had to be frozen on the same day but from different sources i.e. cloning plates and from the original vial from LN_2 storage.

Table 2.3 Cell freezing schedule for H1.8, H3.2 and H3.3

Cell line	Original source	Viability	Vials	Date
H1.8	LN_2	88%	6 at 2×10^6	2/11/09
H1.8	Cloning plate	91%	8 at 2×10^6	3/11/09
H1.8	LN_2	87%	7 at 2×10^6	27/10/09
H3.2	Cloning plate	83%	2 at 2×10^6	19/11/09
H3.2	Cloning plate	86%	3 at 2×10^6	19/11/09
H3.2	LN_2	88%	11 at 4×10^6	19/11/09
H3.3	Cloning plate	95%	6 at 2×10^6	19/11/09
H3.3	LN_2	89%	11 at 4×10^6	27/10/09

The above table indicates what cell lines were frozen, what the original source was, the viability of the cells, how many vials were frozen and the date of the freezing batch.

Chapter 3 Optimisaton of experimental techniques

3.1 Introduction

In order to investigate the relative amount of full-length PrP^C and the C1 fragment in various tissues it is necessary to develop techniques that allow the protein to be visualised and quantified. Techniques for the detection of PrP^{Sc} from brain and peripheral tissue are well established but involve harsh methods that may degrade PrP^C. Methods for the detection of PrP^C are less defined and are made complex because PrP^C is easily degradable. For example PrP^C is sensitive to the enzyme PK whereas PrP^{Sc} is partially resistant (Oesch *et al*., 1985).

Jimenez-Huete *et al*., (1998) investigated ways to differentiate between PrP^C and PrP^{Sc} through the use of different detergents and proteases without the need for PK digestion. After treatment of prion infected human brain tissue with sarkosyl and deglycosylation using PNGase the soluble fraction contained small amounts of PrP^C and an 18 kDa fragment assumed to be C1. The C1 fragment was also detectable in the insoluble PrP^{Sc} fraction of type-1, type-2 CJD and GSS brain samples as well as a fragment ranging from 20 kDa to 22.5 kDa in the various prion diseased models. This truncated fragment could be the C2 fragment which has previously been shown to be present in some CJD cases (Chen *et al*., 1995) and in mouse neuroblastoma cells infected with scrapie (Caughey *et al*., 1991).

Successful detection of full-length PrP^C and the C1 fragment has previously been reported in a variety of species, e.g. sheep, humans, cows and mice (Chen *et al*., 1995, Horiuchi *et al*., 1995, Jimenez-Huete *et al*., 1998, Gatti *et al*., 2002, Nieznanski *et al*., 2005, Laffont-Proust *et al*., 2006, Kuczius *et al*., 2007a,b). PrP^C was also found in sheep and hamster peripheral tissues (Bendheim *et al*., 1992, Horiuchi *et al*., 1995, Moudjou *et al*., 2001). These studies used immunoprecipitation methods to detect PrP^C but did not investigate whether C1 was generated within the periphery as they omitted the deglycosylation step without which C1 at normal expression levels cannot be detected.

Within this section of the thesis methods have been modified with the specific aim of detecting PrP^C and the C1 fragment on a Western blot from brain (3.2.2-3.2.4) and peripheral tissues (3.2.6). Control experiments were carried out to ensure that the C1 fragment is not an artefact produced by degradation of the protein.

3.2 Optimisation of experimental techniques

3.2.1 Detection of PrP^{Sc} from scrapie infected murine brain

To confirm that the available Western blot protocol worked with antibody 6H4 mouse brain tissue from experimentally infected animals was investigated for PrP^{Sc}. Brain material was used from SV or VM mice infected with scrapie strains 22A, 87V and 135A. Figure 3.1 shows immunodetection of PrP^{Sc} after PK treatment with the di glycosylated band at 28 kDa, the monoglycosylated band at 23 kDa and the unglycosylated band at 18 kDa. After PK digestion unglycosylated PrP^{Sc} shifts to 19 kDa.
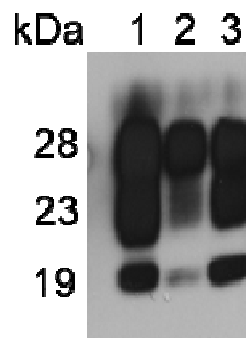
Figure 3.1 PrP^{Sc} detection from scrapie infected animals after PK treatment

Western blot using anti-PrP monoclonal antibody 6H4 to detect PrP^{Sc} in mouse brain extracts. Lane 1 SV brain homogenate (10%) infected with 135A scrapie. Lane 2 VM brain homogenate (10%) infected with 87V scrapie. Lane 3 VM brain homogenate (10%) infected with 22A scrapie. 25 µl of a 1/1000 dilution of 10% brain homogenate was loaded for each sample. 0.5 µl of PK was added to each sample prior to electrophoresis. The diglycosylated form of PrP^{Sc} is at ~ 28 kDa, monoglycosylated PrP^{Sc} at ~ 23 kDa and unglycosylated PrP^{Sc} at ~19 kDa. This experiment was repeated twice (n = 2).

3.2.2 Detection of PrP^C from uninfected mouse brain

Brain tissue was collected from a wild type mouse, from a PrP null mouse and from a PrP hemizygote mouse (cross of null mouse with wildtype) that only has one copy of the PrP gene. This experiment was conducted to compare PrP^C detection limits using 6H4. There is no protein in the sample from the null mouse which is expected. The signal for the di-glycosylated PrP^C fragment in the hemizygote (Figure 3.2, solid line) is considerably reduced compared with the wild-type (Figure 3.2). The faint middle band is most likely monoglycosylated PrP^C. There are two unexpectedly strong bands in the hemizygote (Figure 3.2, bottom dashed lines). The top band may be unglycosylated PrP^C although it appears too strong relative to the diglycosylated band. The second band can as yet not be explained and is not normally seen in mouse PrP preparation but may be glycosylated fragments of PrP.
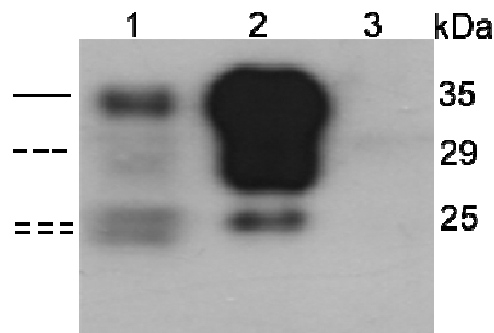
Figure 3.2 Detection of PrP^C from wild type, hemizygote and null mouse brain

Western blot using anti-PrP monoclonal antibody 6H4 to detect PrP^C from wild type, hemizygote and null mouse brain extracts. Lane 1, 10% mouse brain homogenate that only expresses one copy of the PrP gene (hemizygote). Lane 2, 10% mouse brain homogenate (wild type). Lane 3, 10% mouse brain homogenate that has had the PrP gene knocked out (null). 25 µl of a 1/100 dilution of 10% brain homogenate was loaded for each sample. Diglycosylated PrP^C is at ~ 35 kDa, monoglycosylated PrP^C at ~ 29 kDa and the unglycosylated PrP^C at ~ 25 kDa. This experiment was repeated twice (n = 2).

3.2.3 Detection of PrP^C from uninfected ovine brain using two preparation methods

For the detection of PrP^C from uninfected brain tissue two preparation methods were tested, one a simple lysis homogenate (2.1.1) the other including a sucrose gradient purification / enrichment step (2.1.2) and an assessment was made which preparation method was best for ovine brain tissue. Two different antibodies with different sensitivity for PrP^C were used for detection. This experiment verified that P4 was more sensitive than 6H4, but due to its epitope (aa93-99, Figure 3.11) P4 will not detect C1. In the comparison of the two methods the enrichment protocol is not as sensitive as the simple homogenate protocol when it comes to the detection of PrP^C. PrP^C can be detected using the simple homogenate at a higher dilution compared to the enrichment protocol (Figure 3.3).
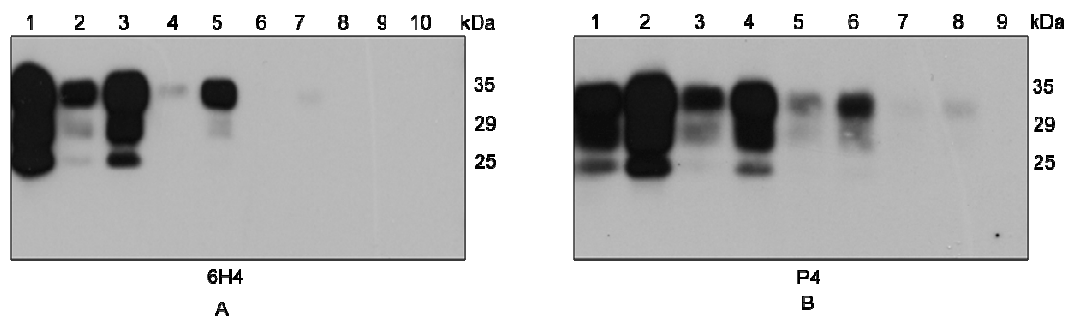
Figure 3.3 Immunodetection of PrP^C from uninfected ovine brain tissue using monoclonal antibodies 6H4 and P4

Western blot using anti-PrP monoclonal antibodies 6H4 (A) and P4 (B) to detect PrP^C from uninfected ovine brain extracts. (A) Lane 1, 3, 5, 7 and 9 contain uninfected ovine brain tissue homogenate (10%) that was prepared using the lysis preparation method (2.1.1). Lanes 2, 4, 6, 8 and 10 contain uninfected ovine brain tissue homogenate (10%) that was prepared using the sucrose gradient purification method (2.1.2) (B) Lanes 1, 3, 5, 7 and 9 contain uninfected ovine brain tissue homogenate (10%) that was prepared using the sucrose gradient purification method. Lanes 2, 4, 6 and 8 contain uninfected ovine brain tissue homogenate that was prepared using the lysis preparation method (2.1.1). Lanes 1 and 2, 1/100 dilution of brain homogenate, lanes 3 and 4, 1/10000 dilution of brain homogenate, Lanes 5 and 6, 1/100000 dilution of brain homogenate, Lanes 7 and 8, 1/1000000 dilution of brain homogenate and lanes 9 and 10, 1/10000000 dilution of brain homogenate. 20µl of each brain homogenate dilution was loaded. The diglycosylated band PrP^C is at ~ 35 kDa, monoglycoyslated PrP^C at ~ 29 kDa and unglycosylated PrP^C at ~ 25 kDa. Each experiment was repeated twice (n = 2).

3.2.4 Detection of PrP^C from uninfected ovine and murine brain tissue

Brain homogenate from Cheviot sheep number 30 x 31, an animal confirmed to be clinically negative and negative for PrP^{Sc} was prepared using the lysis preparation method (2.1.1). A murine brain tissue sample from a 129/Ola mouse was used as a control to compare detection levels of PrP^C between ovine and murine tissues (Figure 3.4). Anti-PrP monoclonal antibody 6H4 was used for the detection of PrP^C.
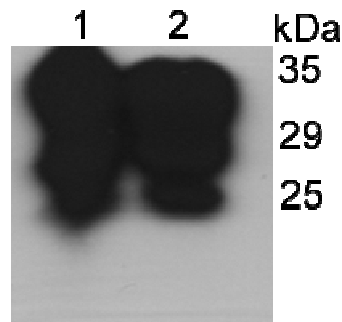
Figure 3.4 Detection of PrP^C from uninfected ovine and murine brain tissue

Western blot using anti-PrP monoclonal antibody 6H4 to detect PrP^C from uninfected ovine and murine brain tissue. Lane 1 contains 10% uninfected mouse brain homogenate (strain 129/Ola). Lane 2 contains 10% ovine uninfected brain tissue homogenate (animal 30 x 31). 20µl of a 1/100 dilution of 10% brain homogenate was loaded. The di-glycosylated band of PrP^C is at ~ 35 kDa, monoglycosylated PrP^C at ~ 29 kDa and unglycosylated PrP^C at 25 kDa. This experiment was repeated twice (n = 2).

3.2.5 PrP^C expression patterns in ovine brain regions

To determine the relative expression levels of PrP^C among different brain regions to aid the decision on which brain region would be best for the analysis of PrP and C1, medulla, cerebellum, frontal cortex and thalamus brain homogenates were analysed. Examples of Western blot analysis and glycoform patterns can be observed in Figures 3.5-3.7.

In Figure 3.5 there are higher signal intensities of PrP^C in all brain regions using antibody BC6. The diglycosylated form of PrP^C in cortex appears to run higher than other brain areas. In the cerebellum, cortex and thalamus there is lower molecular weight bands when antibody BC6 is used for detection. These bands may correspond to degradation products of glycosylated forms of the C1 fragment as the lowest molecular weight band in the cerebellum migrates at aproximately 18 kDa.
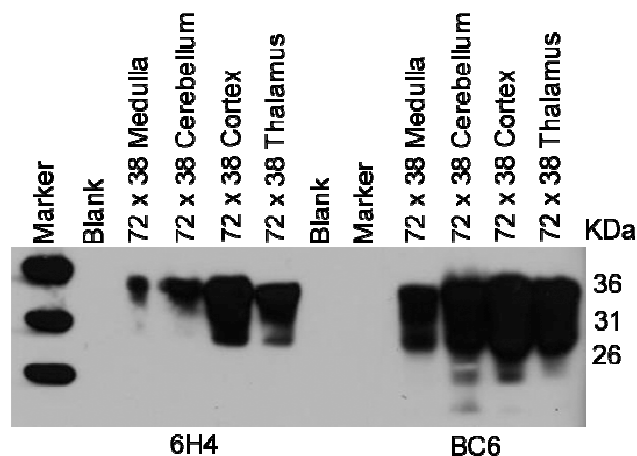
Figure 3.5 PrP glycoform patterns from different brain areas of sheep 72 x 38 using monoclonal antibodies 6H4 and BC6

Western blot using anti-PrP monoclonal antibodies 6H4 and P4 to detect PrP^C from the medulla, cerebellum, cortex and thalamus of an ARQ/ARR sheep. For each brain area 10 µl of a 10% brain homogenate was loaded. The diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa and unglycosylated PrP^C at ~ 26 kDa. The diglycosylated band of PrP in the cortex appears to run higher than the other brain areas this is more noticeable when 6H4 is used for detection. This Western blot demonstrates that BC6 is the more sensitive antibody and that the most dominant glycoform for all brain regions is di-glycosylated PrP^C. This experiment was repeated twice (n = 2).

In Figure 3.6 the signal intensities of PrP^C are higher in the cortex and medulla when antibody 6H4 is used for detection, whereas the signal intensities of PrP^C in the cerebellum and thalamus are higher when antibody P4 is used for detection. Only di-glycosylated forms of PrP^C are observed in the cerebellum and thalamus after 6H4 detection. Again the diglycosylated form of PrP^C in cortex appears to run higher than other brain regions, this might suggest that glycosylation within the cortex PrP^C is different from other brain regions.
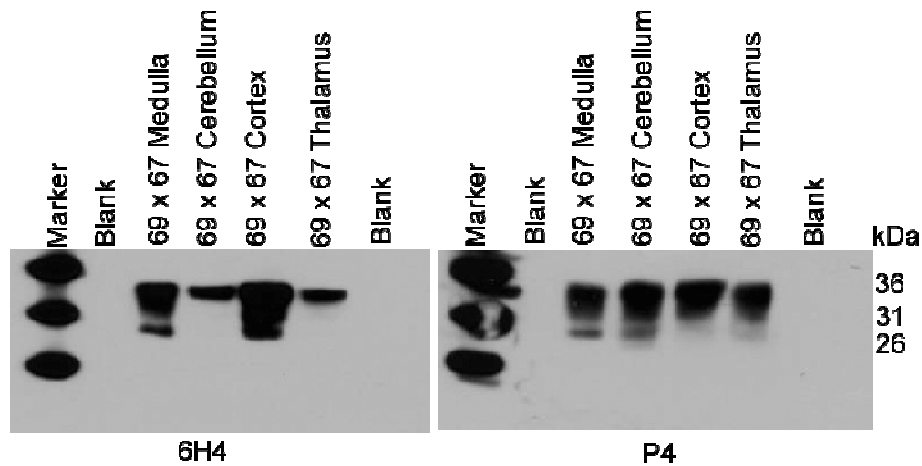
Figure 3.6 PrP glycoform patterns from different brain areas of sheep 69 x 67 using tibodies 6H4 and P4.

Western blot using anti-PrP monoclonal antibodies 6H4 and P4 to detect PrP^C from the medulla, cerebellum, cortex and thalamus of an ARQ/ARQ sheep. For each brain area 10µl of a 10% brain homogenate was loaded. The diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa and unglycosylated PrP^C at ~ 26 kDa. Again the diglycosylated band of PrP^C in the cortex appears to run higher than the other brain areas. This Western blot demonstrates that P4 is more sensitive than 6H4 and that the dominant glycoform for all brain regions is diglycosylated PrP^C. This experiment was repeated twice (n = 2).

In Figure 3.7 PrP^C signal intensities are highest for all brain regions when antibody P4 is used for detection. For all brain areas diglycosylated PrP^C is the most dominant glycycoform when antibody BC6, 6H4 and P4 is used for detection. However when antibody BE12 is used for detection unglycosylated PrP^C is the most dominant glycoform. The difference observed between the different antibodies may be attributed to differences in their epitopes (Figure 3.11).
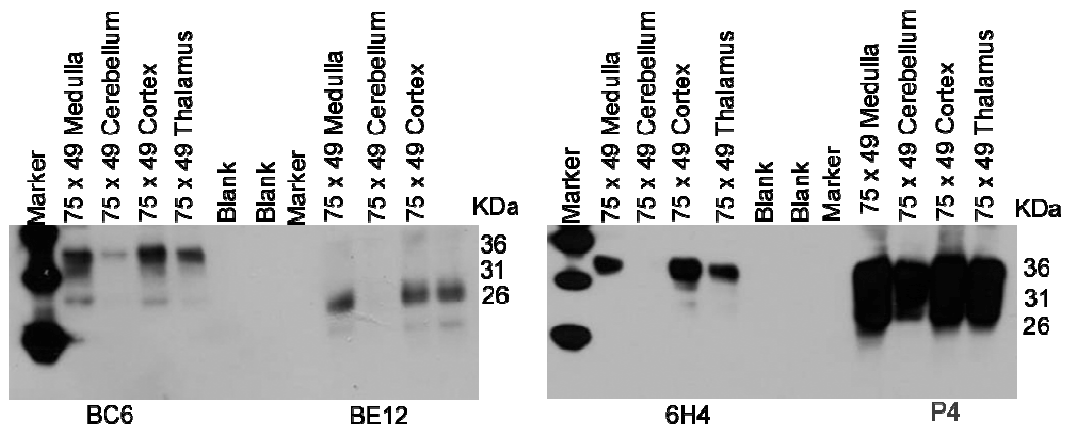
Figure 3.7 PrP glycoform patterns from different brain areas of sheep 75 x 49 using monoclonal antibodies BC6, BE12, 6H4 and P4

Western blots using anti-PrP monoclonal antibodies BC6, BE12, 6H4 and P4 to detect PrP^C from the medulla, cerebellum, cortex and thalamus of an ARR/ARR sheep. For each brain area 10 µl of a 10% brain homogenate was loaded. The diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa and unglycosylated PrP^C at ~ 26 kDa. The diglycosylated band of PrP^C in the cortex appears to run higher than the other brain areas. This Western blot demonstrates that antibody P4 is the most sensitive antibody and that the dominant glycoform for all brain regions is diglycosylated PrP^C except when BE12 is used for detection. When BE12 is used for detection unglycosylated PrP^C is the dominant glycoform. This experiment was repeated twice (n = 2).

3.2.6 PrP^{C} preparation methods for peripheral tissue

As the level of PrP^{C} expression in sheep peripheral tissues is approximately twenty fold less than in the brain (Gossner *et al*., 2009) it was investigated whether PrP^{C} could be detected from peripheral tissues using the crude 10% homogenate preparation method (2.1.1) without the need for purification or immunoprecipitation of the sample. Ovine spleen samples and murine small intestine samples were tested. We found that PrP^{C} could not be detected in ovine spleen using this method when monoclonal antibodies 6H4 and BC6 were used (Figure 3.8).

Unspecific protein binding was detected in the murine small intestine samples (Figure 3.9) when BC6 was used which was confirmed with a sample from null mouse small intestine (Figure 3.9).
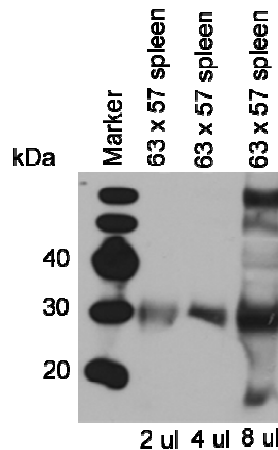
Figure 3.8 Protein detected in ovine spleen homogenate using monoclonal antibody BC6

Western blot using anti-PrP monoclonal antibody BC6 to detect PrP^{C} from sheep 63 x 57 spleen homogenate. 2 μl, 4 μl and 8 μl of a 10% spleen homogenate was loaded. This was equivalent to 20, 40 and 80 μg of total protein as measured using the BCA protein assay. Unspecific binding of protein was detected at ~ 30 kDa. This experiment was repeated twice (n = 2).
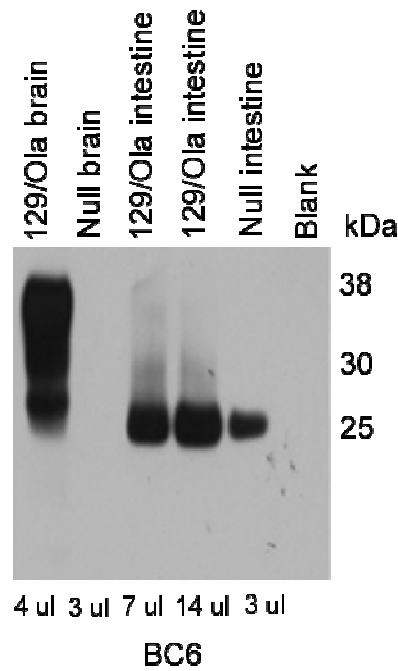
Figure 3.9 PrP detected in murine brain and intestinal tissue using monoclonal antibody BC6

Western blot using anti-PrP monoclonal antibody BC6 to detect PrP^C in the 129/ola brain and 129/Ola intestine. 3 µl, 7 µl and 14 µl of 10% intestinal homogenate was loaded. This was equivalent to 20 µg and 40 µg of total protein. 4 µl of 10% 129/Ola brain homogenate was loaded. This was equivalent to 20 µg of total protein. 3 µl of 10% null brain and null intestinal homogenate was loaded. This was equivalent to 20 µg of total protein as measured using the BCA protein assay. 129/Ola brain was loaded as a positive control. The diglycosylated form of PrP^C is at ~ 26 kDa, monoglycosylated PrP^C at ~ 30 kDa and the unglycosylated PrP^C at ~ 38 kDa. Unspecific protein bands were detected at ~ 25 kDa which is slightly lower than the unglycosylated form of PrP^C of the 129/Ola control. This experiment was run once (n = 1).

The next method that was tested for the detection of PrP^C in peripheral tissues was a purification protocol that had been cited previously (Mohri *et al.*, 1992). This purification method involves the use of potassium chloride and a sucrose gradient (2.1.2). For this experiment ovine spleen samples were used, however PrP^C could not be detected using this method when monoclonal antibody 6H4 was used for detection (data not shown). As these methods were unsuccessful for detecting PrP^C from peripheral tissue, immunoprecipitation using a panel of monoclonal antibodies was investigated (Chapter 6).

3.2.7 PNGase deglycosylation of PrP^C

Optimum conditions for the enzymatic deglycosylation of PrP^C in total brain homogenate were experimentally determined using commercially available PNGase enzyme. Initially 50 μl of 10% brain homogenate was incubated with PNGase for 2 hours at 37°C as recommended by the manufacturers; however these conditions did not allow for complete de-glycosylation of PrP^C although the truncated fragment C1 was detectable (Figure 3.10 A). Decreasing the starting 10% brain homogenate to 25 μl and in some cases 12.5 μl allowed for complete deglycosylation of the protein (Figure 3.10 B).

In Figure 3.10 B there is a strong appearance of the C1 fragment band following deglycosylation although there appears to be no mono-glycosylated PrP^C in the untreated sample. It was previously assumed that prior to PNGase treatment, all of the glycosylated C1 is hiding within the monoglycosylated and unglycosylated full-length PrP which becomes visible after deglycosylation. However this does not appear to be the case. One explanation may be that the PNGase reaction itself, may generate C1 because of contaminants. However this seems unlikely as the C1 fragment does not appear in every PNGase treated sample, this is observed for sheep H30 and H84 (Table 4.1).
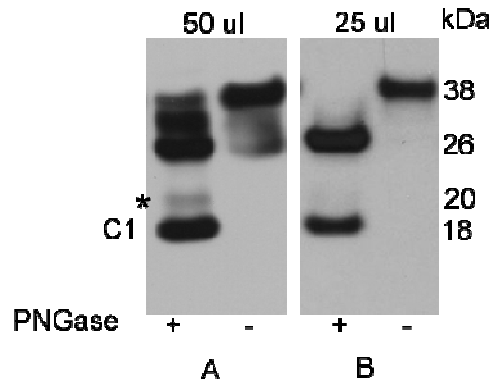
Figure 3.10 Western blots of PNGase treated ovine brain tissue

Western blots using of PNGase deglycosylated PrP^C and undigested PrP^C in uninfected ovine brain material detected using anti-PrP monoclonal antibody 6H4. (A) 50 μl of a 10% brain homogenate was incubated for 2 hours at 37℃ with 1000 units/ml of PNGase. Complete deglycosylation of the protein has not been successful as the di and mono glycosylated bands of PrP^C are still present. An extra band assumed to be the C2 fragment migrates around 20 kDa, and is indicated by * on the blot. (B) 25 μl of a 10% brain homogenate was incubated for 2 hours at 37℃ with 1000 units/ml of PNGase. After altering PNGase conditions there is complete deglycosylation of the protein. 3 μl of 10% brain homogenate was loaded in each lane. The diglycosylated form of PrP^C is at ~ 38 kDa, monoglycosylated PrP^C at ~ 35 kDa and unglycosylated PrP^C at ~ 26 kDa. The C1 fragment is at ~ 18 kDa while the unidentified fragment (*) assumed to be C2 in (A) is at ~ 20 kDa.

3.3 Controls

3.3.1 Antibody mapping

To visualise the C1 fragment on a Western blot an antibody with a C-terminal epitope (between codons 115-230) is required as the C1 fragment is N-terminally truncated. Throughout this thesis monoclonal antibody 6H4 has been used to detect full-length PrP and C1. To confirm that the fragment was indeed C1, 6H4 was stripped from the membrane and the membrane re-probed with N-terminal antibody P4. When the membrane is probed with 6H4 the C1 band is present, however when it is probed with P4 the C1 band is not present (Data not shown). This indicates that the C1 fragment does not contain the P4 epitope and is as expected N-terminally truncated.

To investigate whether the amount of full-length PrP and C1 visualised on a Western blot could be influenced by the choice of antibody, BC6 was also used to detect full-length PrP and the C1 fragment. BC6 gave a stronger signal for both full-length PrP and C1 when used at the same concentration as 6H4. The epitopes for antibodies 6H4, P4 and BC6 is shown in Figure 3.11.
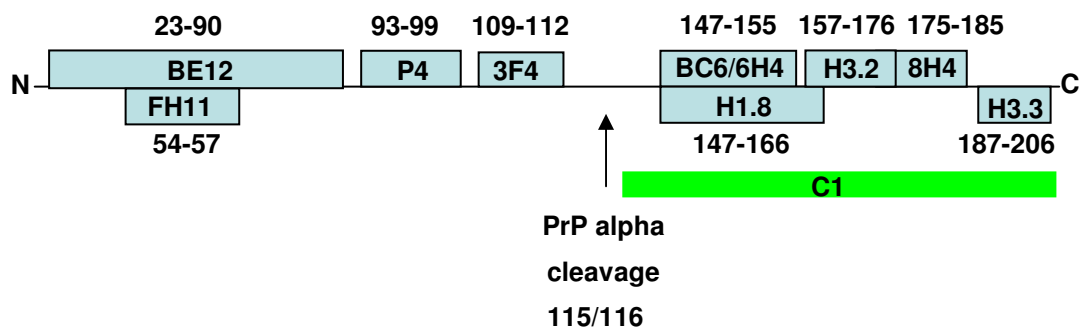
Figure 3.11 Antibody map

Schematic representation of ovine PrP with the location of the epitopes used to generate and recognise the antibodies BE12, FH11, P4, 3F4, 6H4, BC6, 8H4, H1.8, H3.2 and H3.3. Location of the alpha cleavage site and the C1 fragment is shown. The epitope for 3F4 is only available for humans and hamster but because this antibody was used in this thesis it has been included on the diagram.

3.3.2 Degradation experiments and storage conditions

It is possible that C1 is also a degradation product of full-length PrP^C generated at the time of or shortly after *post-mortem*. It may also be generated when frozen tissue is thawed and during PNGase treatment. To investigate the effect of storage and handling conditions on the amount of full-length PrP and C1 in tissue samples, a number of control experiments were carried out. For most of the experiments in this thesis, tissue was taken from the outside of the brain material as this is the area that defrosts first and is also clearly recognisable as cortex.

Tissue from the inside of a frozen sheep brain (frontal cortex) and tissue from the outside of the same frozen sheep brain were compared. After deglycosylation and Western blot analysis there was little difference in the amount of full-length PrP and C1. (Figure 3.12 lanes 1 and 2). This experiment was repeated twice with reproducible results.

Although measures were taken to avoid *post-mortem* material being at room temperature for longer than necessary (as given by the procedure), material was not always frozen instantly. To test the effect of this, 0.2 g of tissue (not mazerated or homogenised) from a sheep brain (frontal cortex) was incubated at room temperature for 16 hours without the presence of protease inhibitors. This sample was deglycosylated along with tissue from the same animal that had not been subject to 16 hours defrost. After Western blot analysis there was little difference in the amount of full-length PrP^C and C1 between the experimental and control tissue (Figure 3.12 lanes 3 and 4).

To test whether freeze thawing could influence the ratio between full-length PrP^C and C1 the final control experiment involved taking 0.2 g brain tissue (frontal cortex) and subjecting it to four freeze thaw cycles (Tissue defrosted at room temperature and refrozen at -20°C). As a control, brain tissue from the same animal was used which was not subject to freeze thaw conditions. After deglycosylation and Western blot analysis there was little difference in the amount of full-length PrP^C and C1 between the experimental and control tissue (Figure 3.12 lanes 5 and 6).
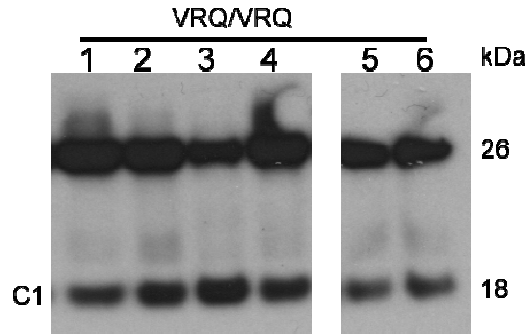
Figure 3.12 Ovine PrP^C degradation experiments (non-continous gel)

Western blot of PNGase deglycosylated PrP^C in uninfected ovine brain material detected using anti-PrP monoclonal antibody 6H4. 25 µl of a 10% sheep brain homogenate was incubated for 2 hours at 37°C with 1000 units/ml of PNGase. Lane 1 contains a homogenate that was prepared from the inside of a sheep brain tissue. Lane 2 contains a homogenate that was prepared from the outside of the same sheep brain tissue as shown in lane 1. Lane 3 contains a homogenate that was prepared from brain tissue that was left at room temperature for 16hrs in the absence of protease inhibitors. Lane 4 contains a homogenate from the same animal shown in lane 3 but which was not left at room temperature for 16 hours. Lane 5 contains a brain homogenate that was subject to 4 x freeze thaw cycles. Lane 6 contains a brain homogenate from the same animal in lane 5 that was not subject to 4 x freeze thaw cycles. 5 µl of a 10% brain homogenate was loaded for each sample. The unglycosylated form of PrP^C is at ~ 26 kDa and the C1 fragment at ~ 18 kDa. This experiment was repeated twice (n = 2).

3.3.3 Distinguishing PrP^C from PrP^{Sc}

PrP^{Sc} can easily be distinguished from PrP^C when it is treated with proteinase K (PK). PrP^{Sc} is partially resistant to this protease and when it is treated with Proteinase K it is truncated to leave a 19-30 kDa protein pattern. When PrP^C is treated in the same way the protein is completely digested. Thus Proteinase K is used to distinguish between infected and uninfected samples during immunoblotting as only PrP^{Sc} will be detected (Figure 3.13). In Figure 3.13 there appears to be four bands present, this is not usually the case as three bands corresponding to diglycoyslated, monoglycosylated and unglycosylated PrP are usally detectable. It is possible that one of the extra bands could be glycosylated C1. However, after longer exposure there appears only to be three bands present (Data not shown).
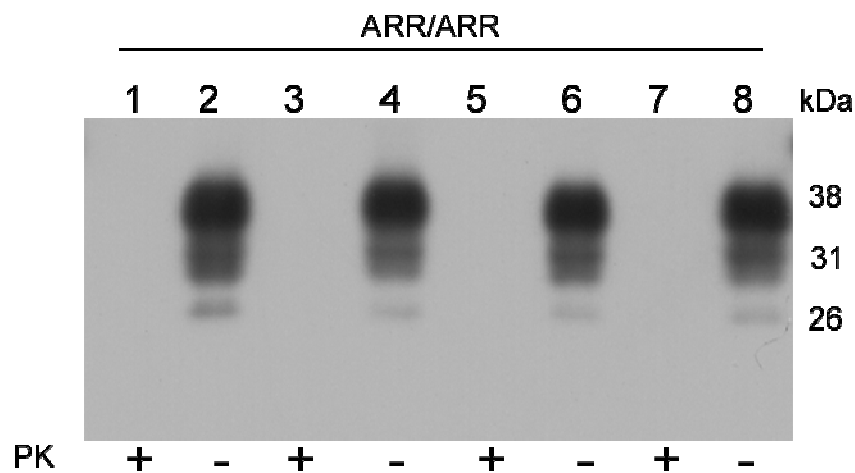
Figure 3.13 Ovine brain samples treated with and without proteinase K

Western blot of ovine brain samples treated with and without proteinase K and detected using anti-PrP monoclonal antibody 6H4. Total ovine brain protein was treated for 1 hour with at 37°C with 5mg/ml of PK. Samples were denatured at 100°C for 10 minutes and electrophoresis carried out. In the lanes indicated with +PK no PrP^{Sc} was detected. This indicates that these samples were uninfected. 1/100000 dilution of 10% brain homogenate was loaded for each sample. Diglycosylated PrP^C is at ~ 38 kDa, monoglycosylated PrP^C at ~ 31 kDa and unglycosylated PrP^C at ~ 26 kDa. This experiment was repeated twice (n = 2).

3.4 Discussion

The main aim of this thesis is to analyse the amount of full-length PrP and the C1 fragment in various tissues to examine their role on TSE susceptibility. This chapter describes the effort in optimising PrP preparation methods for the detection of full-length PrP and truncated PrP fragment C1 and the essential controls to show that the experimental approach is sound.

Two methods were used for the preparation of PrP^C from ovine and murine tissues (2.1.1-2.1.2). Results from (Figure and 3.3 A and B) show that the signal detection levels of PrP^C in ovine brain were stronger using the crude homogenate preparation method than the sucrose preparation method. The reason behind this may be that some of the PrP is degraded during the sucrose method as there is an enrichment and purification step used in this protocol. Unfortunately, we were unable to detect PrP in ovine spleen and murine small intestine (Figures 3.8, 3.9) using the crude or purification method therefore immunoprecipitation experiments were carried out (Chapter 6). The C1 fragment was also successfully detected in ovine brain tissue after some modification of the protocol (Figure 3.10).

The amount of PrP^C in different brain areas was also analysed along with the corresponding glycoform patterns (Figures 3.5-3.7). The main reason behind these experiments was to determine which brain area should be used in future experiments. All available antibodies detected diglycosylated PrP^C the most. Although BE12 did detect unglycosylated PrP^C as the dominant isoform this was in only one animal, and in other cases this antibody was not as sensitive as 6H4. The frontal cortex and the medulla gave the strongest signal when antibody 6H4 was used for detection. The frontal cortex was chosen for analysis in the ovine (Chapter 4) and murine (Chapter 5) deglycosylation experiments as the frontal cortex was available for all animals used in the study.

There is little difference in the level of PrP^C detection between ovine and murine brain samples when monoclonal antibody 6H4 is used (Figure 3.4). This was an important result as later experiments were carried out on murine models expressing

murine, ovine and bovine PrP (Chapter 5) therefore it was important to determine the normal levels of murine PrP^C before beginning these experiments.

Results from the degradation experiments indicate that both full-length PrP and the C1 fragment are very stable as very little degradation is observed after four freeze thaw cycles when compared to the control sample (Figure 3.12). If degradation occurs it appears to be roughly equal for both full-length PrP and the C1 fragment. The importance of the freeze thawing experiments was to see if results could be influenced by certain storage and handling conditions of the tissue sample. Most of the tissue used in the experiments was taken fresh and frozen immediately at -70°C. However, archived tissue which had been frozen for a number of years was also used. This archived tissue is most likely to have been thawed previous to our experiments. The results indicate that freeze thawing of tissue samples does not lead to immediate degradation of either full-length PrP or C1 or spontaneous production of C1. These experiments indicate that our results have not been influenced by freeze thawing of sample tissue. We repeated this experiment twice on two separate samples which had not been subject to freeze thawing previously with reproducible results.

By law, human brain samples can not be taken for a number of hours after death therefore to try and mimic human post-mortem conditions within the laboratory, tissue without the presence of protease inhibitors was left for 16 hours at room temperature. Remarkably there was little degradation compared to the control sample (Figure 3.12) which had not been left for 16 hours at room temperature. This is likely not to be the case when the tissue is homogenised. We repeated this experiment twice on two separate samples with reproducible results. Some lamb tissue was collected a number of hours after the animal had died. These control experiments indicate that these results may not be influenced as much by the conditions of the tissue as would have been predicted. Overall our degradation experiments indicate that PrP and the C1 fragment are relatively stable within tissues and are not easily degraded by various storage and handling conditions. These experiments also provide evidence that the C1 fragment is not a degradation product due to harsh handling and long

term storage conditions and is a true cleavage product. These data are also relevant when comparisons are made between the analysis of this thesis and publications on alpha cleavage.

Chapter 4 Alpha cleavage of PrP in ovine brain

4.1 Introduction

Sheep with different *PRNP* genotypes are differentially susceptible to scrapie disease. To investigate whether the amount of alpha cleavage correlates with scrapie disease, C1 as a percentage of total PrP^C was measured in sheep with different *PRNP* genotypes, and therefore different scrapie susceptibility.

PRNP genotypes in sheep are grouped in accordance to their amino acids at codons 136, 154 and 171 of ovine PrP. Polymorphisms at these three amino acid positions are known to confer resistance or susceptibility to scrapie (for more details see Chapter 1). Disease outbreaks appear to be dependent on allele frequency and breed as the VRQ and ARQ alleles confer different susceptibility when expressed in different sheep breeds. *PRNP* genetics also applies to incubation period differences. For example New Zealand Cheviot VRQ/VRQ sheep have shorter incubation periods compared to Cheviot sheep held at the Neuropathogenesis Division (NPD/NPU) Roslin, after experimental challenge with SSBP/1 (Houston *et al*., 2002b).

Most available data allows the suggestion that polymorphisms that are within close structural proximity have similar effects on disease susceptibility. For example codons 136 and 171 are structurally close to each other (Haire *et al*., 2004) and are both associated with classical scrapie (Goldmann *et al*., 1990,1991a). Codons 141 and 154 are even closer to each other and are both associated with atypical scrapie (Benestad *et al*., 2003, Moum *et al*., 2005, Colussi *et al*., 2008). As the alpha cleavage site is located within the unstructured region of PrP it is possible that this region is close to one or all of these polymorphisms which could therefore influence PrP processing by altering the enzyme binding site, making it more or less accessible to cleavage.

At present there is insufficient data within the literature about the processing of alpha cleavage and its biological consequences. Previous studies of the proteolytic processing of PrP have used both *in vivo* and *in vitro* approaches. These studies have

been carried out on a variety of species, such as sheep, humans, cows, mice and pigs (Chen *et al*., 1995, Horiuchi *et al*., 1995, Jimenez-Huete *et al*., 1998, Gatti *et al*., 2002, Nieznanski *et al*., 2005, Tveit *et al*., 2005, Laffont-Proust *et al*., 2006, Kuczius *et al*., 2007a,b).

Residues 106-126 have been suggested to be involved in the conversion process of the prion protein (Forloni *et al*., 1993,1994, Gu *et al*., 2002). The C1 fragment contains only residues 115-126 of this domain; therefore it may be that cleavage within this region affects the ability of PrP^C to be an effective template for conversion to PrP^{Sc}. To gain further understanding of the proteolytic processing this chapter presents the analysis between the levels of cleavage product (C1) in the cortex and genotypes susceptible to classical scrapie.

4.2 Results

4.2.1 Quantification of the C1 fragment as a percentage of total PrP^C

The quantification of C1 as a percentage of total PrP^C was calculated in the frontal cortex of sheep with different *PRNP* genotypes. This region was chosen for various reasons; firstly, this brain area was available for all sheep that were analysed; secondly, this area is always affected at the terminal disease time point (Jeffrey *et al*., 2001). Thirdly, more PrP was consistently detected via immunoblotting in the frontal cortex compared to other brain regions (Chapter 3).

Frontal cortex tissue was collected from sheep of two breeds (Cheviot and Poll Dorset) and seven *PRNP* genotypes (based on the codons 136, 154 and 171) (see Appendix 1, Table I). Each genotype group contained at least four animals. The largest group was the VRQ/VRQ genotype, which contained ten animals as it included both Poll Dorset and Cheviot sheep. Brain homogenates were prepared according to the PrP^C lysis preparation protocol (2.1.1) and deglycosylated using the enzyme PNGase. PrP^C and the truncated C1 fragment were detected by Western blotting using monoclonal antibody 6H4. For some samples the assay was repeated

with monoclonal antibody BC6. For each sheep at least two separate tissue extractions were prepared and each sample was assayed twice.

After PNGase treatment to remove the carbohydrates, PrP^C was detected in most cases as two distinct bands, unglycosylated full-length PrP^C and unglycosylated C1. However, a band of around 20 kDa was present in some of the samples (Figure 4.2 B, C; Figure 4.3 A, B and C), this was often but not always accompanied by some monoglycosylated full-length PrP^C. The 20 kDa band may be present due to other proteolytic cleavage, such as β cleavage in which the 20 kDa band represents the C2 fragment of PrP^C.

C1 as a percentage of total detectable PrP^C was measured after deglycosylation. The total signals of PrP^C consisting of (full-length PrP^C, C2 and the C1 fragment) and the C1 fragment was defined as 100% and C1 was calculated as a percentage of the combined signal. All results in this thesis will be shown as percentage of total detectable PrP^C. The signal quantification was calculated as a percentage so that a direct comparison could be made to previously published data (Kuczius *et al*., 2007a). Only the C1 fragment was measured as the N1 fragment that is generated in a 1:1 ratio with C1 was not detected with 6H4 or BC6 antibodies. It was also not detected in any of the samples when monoclonal antibody P4 was used suggesting a short half-life. Each sample was loaded with and without PNGase to show that glycosylated forms of PrP^C were present in each sample. However in many cases unglycosylated and monoglycosylated forms of PrP^C are not detected with 6H4.

Based on Molecular weight calculations it can be assumed that the C1 fragment will migrate to the same position as unglycosylated PrP^C which could explain why the C1 band is not detectable on its own. Alternatively, the C1 fragment itself may be di-mono and unglycosylated, each band under the detection limit becoming visible when after deglycosylation the band shift to the same position. It has been observed in a few high expression models with antibody BC6 that di, mono and unglycosylated forms of C1 are detectable before PNGase is applied (Figure 5.9 C).

However, in cases where the unglycosylated band of PrP^C is not detected either, it is difficult to explain the absence of the glycosylated C1 fragment. Although it is possible that C1 is produced during PNGase digestion, it is rather unlikely to be of importance as samples with undetectable C1 were observed regularily and prolonged incubation with PNGase did not increase C1 percentage (Table 4.1).

The average percentage of C1 in all brain samples was around 20-30% with the exception of the ARQ/ARQ and VRQ/ARQ animals that had an average C1 percentage of 16 and 17% respectively. Two Poll Dorest VRQ/ARQ animals had 0% C1, whereas New Zealand Cheviot VRQ/ARQ animals had up to 44% C1. After statistical analysis there was no significant difference between Poll Dorset and New Zealand Cheviots within this genotype group. Figure 4.4 shows a representation of the ratio between C1 and total PrP^C in relation to the different genotypes reflecting average results for each group. The mean percentage values C1 for all animals are presented in Table 4.2.
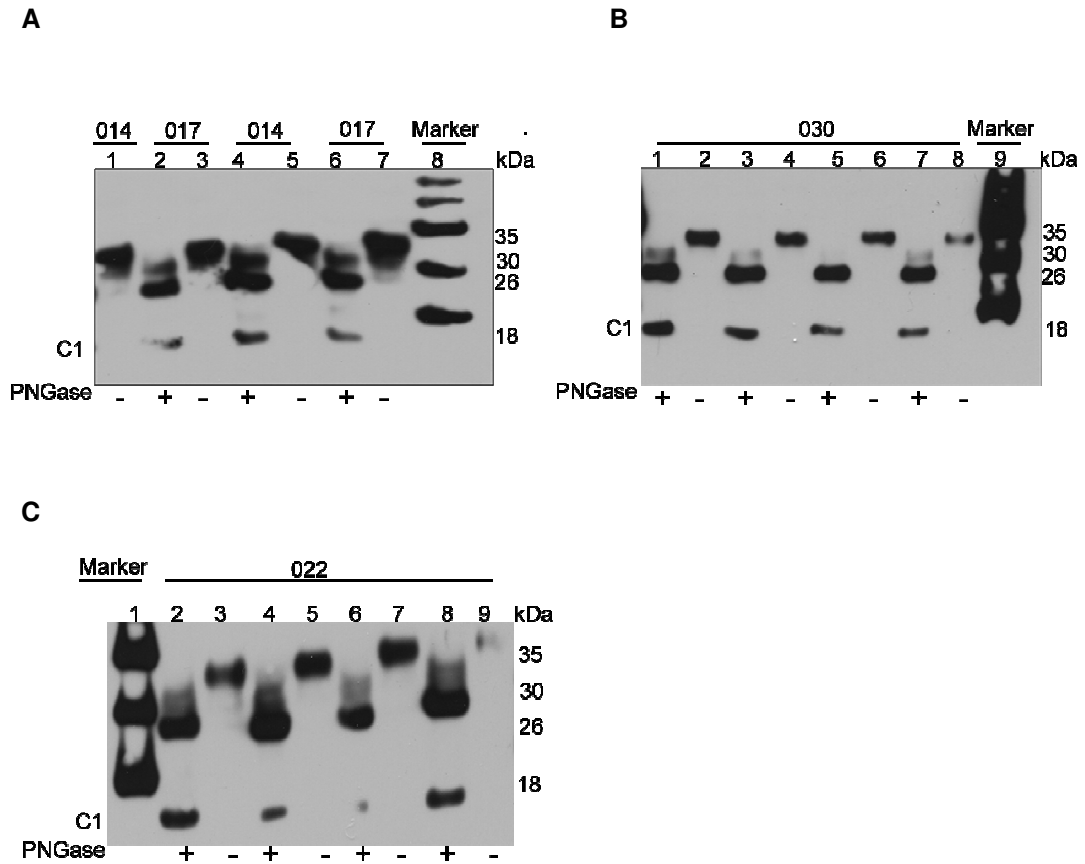
Figure 4.1 PrP^C and C1 detected from ARR/ARR homozygotes using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C in Cheviot sheep no. 014 and no. 017 (ARR/ARR homozygotes). 5 µl of 10% brain homogenate was loaded in each lane. The majority of PrP^C is diglycosylated (lane 1, 3). Remaining monoglycosylated PrP^C was occasionally seen after PNGase treatment (lane 2, 4, 6). (B) Western blot of PNGase deglycosylaed and undigested PrP^C in Cheviot sheep 030 (ARR/ARR homozygote). 5 µl of 10% brain homogenate was loaded in each lane. (C) Western blot of PNGase deglycosylated and undigested PrP^C in Cheviot sheep no. 020 (ARR/ARR homozygote). 5 µl of 10% brain homogenate was loaded in each lane. For Blot A-C the diglycosylated form of PrP^C was at ~ 35 kDa, monoglycosylated PrP^C at ~ 30 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. Each experiment was repeated twice (n = 2).
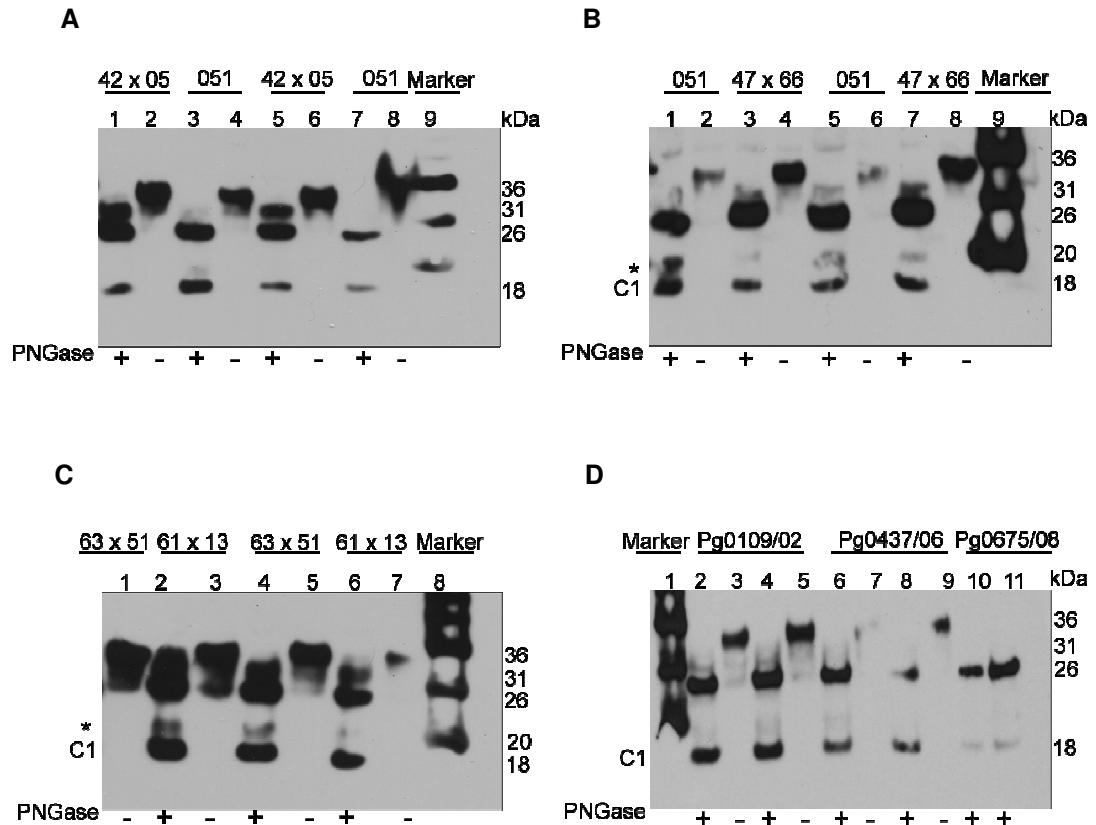
Figure 4.2 PrP^C and C1 detected from various heterozygote and homozygote animals using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C in Cheviot sheep no. 42 x 05 (AHQ/ARR heterozygote) and no. 051 (ARR/ARR homozygote). The majority of PrP^C is diglycosylated. Remaining monoglycosylated PrP^C was occasionally observed after PNGase treatment (lane 1 and 5). (B) Western blot of PNGase deglycosylated and undigested PrP^C in Cheviot sheep no. 050 (AHQ/ARR heterozygote) and no. 47 x 66 (AHQ/ARQ heterozygote). An extra band above C1 (most likely to be the C2 fragment) can be visualised in these samples (Indicated by * on blot) this band migrates at ~ 20 kDa. (C) Western blot of PNGase degylcosylated and undigested PrP^C in cheviot sheep no. 63 x 51 (AHQ/ARQ heterozygote) and no. 61 x 13 (ARQ/ARR heterozygote). An extra band above C1 (most likely to be the C2 fragment) can be visualised in these samples (Indicated by * on blot) this band migrates at ~ 20 kDa. (D) Western blot of PNGase degylcosylated and undigested PrP^C in New Zealand cheviot sheep no. Pg0109/02 (VRQ/ARQ heterozygote), no. Pg437/06 (VRQ/ARQ heterozygote) and no. Pg0675/08 (VRQ/ARQ heterozygote). For Western blots A-D the diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C is at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 µl of 10% brain homogenate was loaded in each lane for Western blots A-D. Each experiment was repeated twice (n = 2).
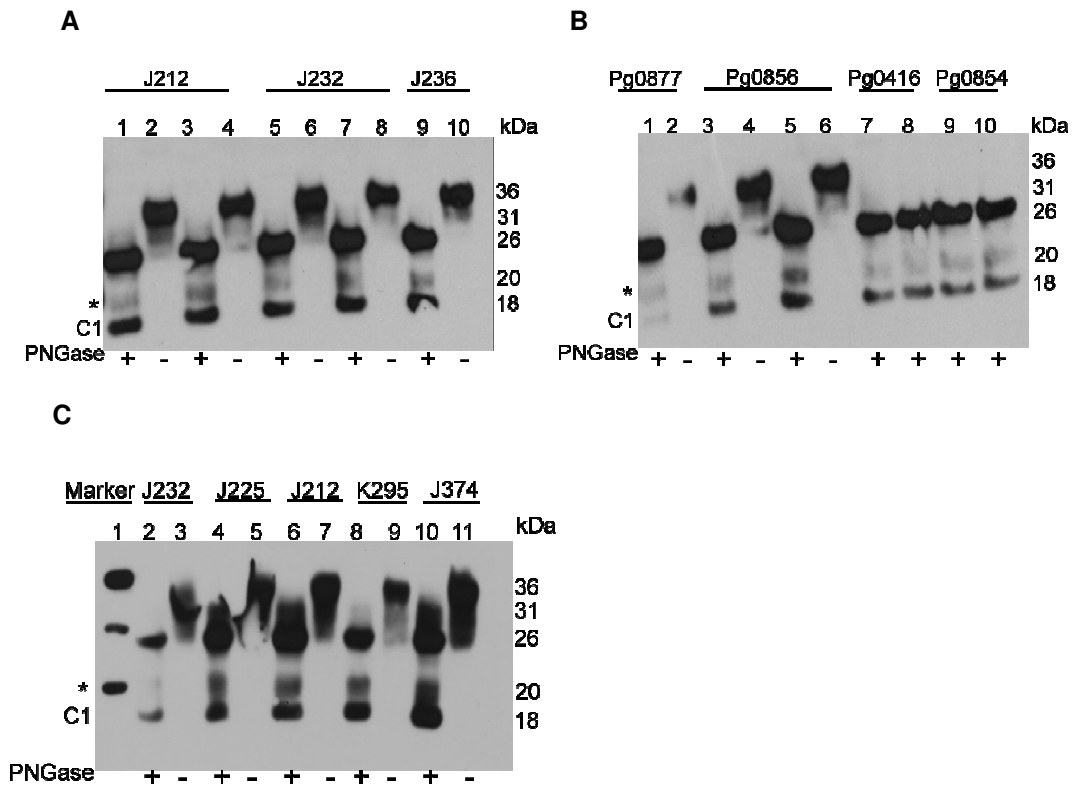
Figure 4.3 PrP^C and C1 detected from VRQ/VRQ and ARQ/ARQ homozygotes using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C in Poll Dorset sheep no. J212, no. J232 and no. J236. All these animals are VRQ/VRQ homozygotes. An extra band above C1 (most likely to be the C2 fragment) can be visualised in these samples (Indicated by * on blot). This band migrates at ~ 20 kDa. Unfortunately there is an air bubble in lane 9 on the C1 fragment; this result was not included in the overall analysis. (B) Western blot of PNGase deglycosylated and undigested PrP^C in Cheviot sheep no. Pg0877, no. Pg0856, Pg0416 and no. Pg0854. All these animals are ARQ/ARQ homozygotes. An extra band above C1 (most likely to be the C2 fragment) can be visualised in all samples (Indicated by * on the blot). This band migrates at ~ 20 kDa. (C) Western blot of PNGase deglycosylated and undigested PrP^C in Poll Dorset sheep no. J232, no. J225 and no. J212 and Cheviot sheep K285 and J374. All these animals are VRQ/VRQ homozygotes. An extra band above C1 (most likely to be the C2 fragment) can be visualised in all these samples (indicated by * on the blot). For Western blots A-C the diglycosylated form of PrP^C is at ~ 36 kDa, the monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 μl of 10% brain homogenate was loaded in each lane for Western blots A-C. Each experiment was repeated twice (n = 2).
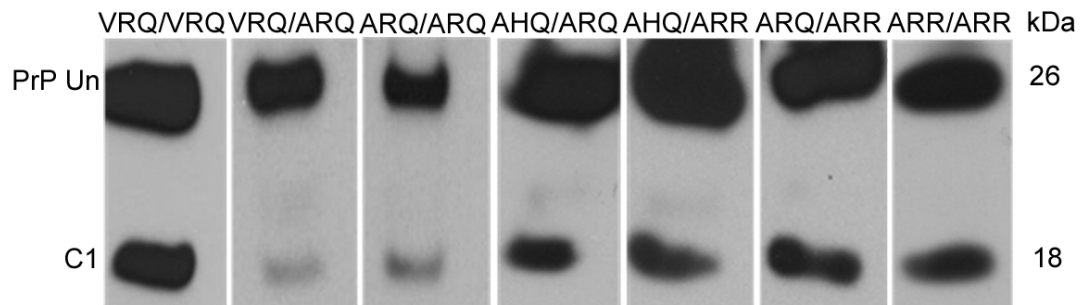
Figure 4.4 Representative Western blots of alpha cleavage profiles from various *PRNP* genotypes using monoclonal antibody 6H4 (montage of different gels)

Western blot analysis of deglycosylated PrP^C from sheep brain tissue detected using monoclonal antibody 6H4. Full-length unglycosylated PrP^C was detected at ~26 kDa and the C1 fragment at ~18 kDa. 5 µl of 10% brain homogenate was loaded for each sample.

Table 4.1 Densitometry data for animals used in genotype study (Frontal cortex)

Genotype	Animal no	Breed	C1: total PrP^C Result 1	C1: total PrP^C Result 2
VRQ/VRQ	J212	Poll Dorset	36:64	32:68
VRQ/VRQ	J225	Poll Dorset	41:59	39:61
VRQ/VRQ	J232	Poll Dorset	44:56	30:70
VRQ/VRQ	J236	Poll Dorset	37:63	28:72
VRQ/VRQ	J255	Poll Dorset	33:67	26:74
VRQ/VRQ	J368	Cheviot	23:77	21:79
VRQ/VRQ	J374	Cheviot	31:69	27:73
VRQ/VRQ	K295	Cheviot	31:69	28:72
VRQ/VRQ	K310	Cheviot	33:67	32:68
VRQ/VRQ	K320	Cheviot	36:64	35:65
ARQ/ARQ	69 x 67	Cheviot	34:66	24:76
ARQ/ARQ	Pg0416	NZ Cheviot	23:77	18:82
ARQ/ARQ	Pg0433	NZ Cheviot	14:86	8:92
ARQ/ARQ	Pg0854	NZ Cheviot	21:79	19:81
ARQ/ARQ	Pg0856	NZ Cheviot	32:68	20:80
ARQ/ARQ	Pg0877	NZ Cheviot	24:76	22:88
VRQ/ARQ	H30	Poll Dorset	0:100	0:100
VRQ/ARQ	H41	Poll Dorset	12:88	3:97
VRQ/ARQ	H50	Poll Dorset	25:75	18:82
VRQ/ARQ	H84	Poll Dorset	0:100	0:100
VRQ/ARQ	Pg0109	NZ Cheviot	44:56	44:56
VRQ/ARQ	Pg0437	NZ Cheviot	43:57	28:72
VRQ/ARQ	Pg0675	NZ Cheviot	6:94	4:96
VRQ/ARQ	Pg0490	NZ Cheviot	27:73	26:74
VRQ/ARQ	Pg1226/04	NZ Cheviot	28:72	19:81
AHQ/ARQ	47 x 66	Cheviot	32:68	23:77
AHQ/ARQ	59 x 89	Cheviot	25:75	16:84
AHQ/ARQ	63 x 51	Cheviot	26:74	22:78
AHQ/ARQ	69 x 24	Cheviot	26:74	25:75
AHQ/ARR	050	Cheviot	33:67	23:77
AHQ/ARR	42 x 05	Cheviot	32:68	25:75
AHQ/ARR	47 x 29	Cheviot	31:69	25:75
AHQ/ARR	71 x 58	Cheviot	31:69	29:71
ARQ/ARR	43 x 39	Cheviot	36:64	13:87
ARQ/ARR	53 x 57	Cheviot	20:80	10:90
ARQ/ARR	61 x 13	Cheviot	22:78	18:82
ARQ/ARR	72 x 38	Cheviot	33:67	28:72
ARQ/ARR	74 x 00	Cheviot	28:72	22:78
ARR/ARR	014	Cheviot	17:83	12:88
ARR/ARR	017	Cheviot	24:76	13:87
ARR/ARR	022	Cheviot	33:67	23:77
ARR/ARR	030	Cheviot	32:68	26:74
ARR/ARR	051	Cheviot	26:74	26:74
ARR/ARR	75 x 49	Cheviot	35:65	33:67

Table showing densitometry data for all sheep used in genotype study. C1 is calculated as a ratio of total PrP^C and shown in the format C1: total PrP^C. Each sample was tested twice (shown as result 1 and result 2).

These results show that PrP alpha cleavage occurs in all seven ovine genotypes that were analysed (Figure 4.4). VRQ homozygote animals contained the highest percentage of C1 when 6H4 was used for detection. To ensure that these results were not antibody specific monoclonal antibody BC6 was used on five VRQ/VRQ samples. Western blot detection with antibody BC6 resulted in similar C1 percentage values with 6H4 detection (Figure 4.5). High percentages of C1 were found in five Cheviot animals of the VRQ/VRQ genotype. To confirm that these results were not specific for this breed, five Poll Dorset VRQ/VRQ animals were collected and tested for the amount of C1. These five Poll Dorset VRQ/VRQ sheep had C1 percentages which were comparable to the VRQ/VRQ Cheviot sheep.
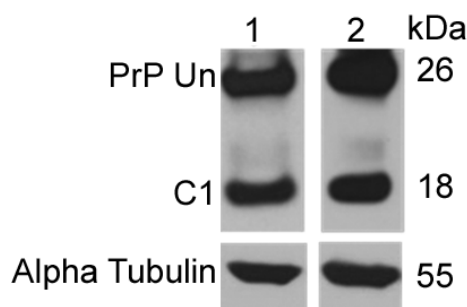
Figure 4.5 Alpha cleavage profiles from VRQ homozygote (montage of different gels) Lane 1 Western blot analysis of deglycosylated PrP^C from VRQ/VRQ sheep brain homogenate (10%) detected using monoclonal antibody 6H4. Lane 2 Western blot analysis of deglycosylated PrP^C from VRQ/VRQ sheep brain homogenate (10%) detected using monoclonal antibody BC6. Alpha tubulin was used as a loading control. 5 µl of 10% brain homogenate was loaded for each sample. Unglycosylated PrP^C was at ~ 26 kDa, the C1 fragment at 18 kDa and the alpha tubulin loading control at ~ 55 kDa. This experiment was repeated twice (n = 2).

4.2.2 Statistical analysis (REML)

Analysed data from Western blots were available for replicate samples of brain tissue from 44 healthy sheep (Table 4.1). The data were unbalanced in respect to some factors i.e. age, sex and breed therefore some comparisons between genotype could not be made within the same breed and sex group. It was possible to collect a more informative summary of the data by providing assumptions about certain effects. For example, all genotypes that were analysed are found in Cheviot and Poll Dorset sheep therefore the means for genotype adjusted for breed and sex were estimated on the condition that differences between genotypes are the same in all breeds and between males and females.

These assumptions allow genotype differences to be estimated both directly from comparisons of observed means within breed/sex combinations and indirectly from comparisons of means between breed/sex comparisons. To fit a model that included age, breed and genotype the data was analysed using REML (residual maximum likelihood). A linear mixed model with random variation between animals and between repeat observations within animals was used to estimate means and standard errors of the C1 fragment. The parameters of the model were estimated using REML directive in Genital 10th edition.

Approximate statistical significance of differences between genotype means was evaluated using Student's t distribution with 41 degrees of freedom, calculating t from the difference between any two genotype means and its corresponding standard error. For example, ARR/ARR v. VRQ/VRQ gives t = (25.47-29.72)/6.78 = -0.63, p >0.53. Table of Means and corresponding standard errors can be observed in Table 4.2.

After statistical analysis between the genotype groups a significant difference was found between ARQ/ARQ homozygotes and VRQ/VRQ homozygotes (p<0.05) and between VRQ/ARQ heterozygotes and VRQ/VRQ homozygotes (p<0.01). A comparison of the genotype means using the student t-test can be observed in Table 4.3.

All differences between genotypes in the same breed and sex are not statistically significantly different from zero (p>0.05) except for the VRQ/ARQ v. VRQ/VRQ comparison in Poll Dorset females p<0.01. The mean for VRQ/ARQ in Poll Dorset females is strikingly low with 2 out of the 6 animals observed with zero C1. The same comparison in Cheviot females is p>0.22.

The REML analysis was also used to estimate the variance of C1 between repeat observations on the same animal. The pooled variance of C1 between repeat observations on the same animal was calculated as 26.77 with a standard deviation of 5.17. This is somewhat smaller than the standard deviation between observations made on different animals (of the same breed, sex, genotype and age) estimated to be 8.95.

Although a significant difference was found between VRQ/VRQ homozygotes and VRQ/ARQ heterozygotes and VRQ/VRQ homozygotes and ARQ/ARQ homozygotes no significant correlation was found between the amont of C1 and incubation period data (SSBP/1) after analysis of the genotype means $R^2 = 0.0058$ (Figure 4.6). This result therefore suggests that there is no linkage between the amount of C1 and scrapie disease susceptibility.

Table 4.2 C1 Mean percentage for each Genotype averaged equally over Breed and Sex adjusted to the mean age of 43 months

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Genotype	AHQ/ARQ	AHQ/ARR	ARQ/ARQ	ARQ/ARR	ARR/ARR	VRQ/ARQ	VRQ/VRQ
	20.46	27.16	15.87	22.27	25.47	16.72	29.72

Standard errors of differences between pairs

Genotype AHQ/ARQ	1	*					
Genotype AHQ/ARR	2	6.40	*				
Genotype ARQ/ARQ	3	5.95	5.60	*			
Genotype ARQ/ARR	4	5.56	5.66	4.66	*		
Genotype ARR/ARR	5	5.96	5.75	5.58	5.11	*	
Genotype VRQ/ARQ	6	6.71	6.35	4.96	6.13	6.63	*
Genotype VRQ/VRQ	7	7.21	6.49	5.15	6.53	6.78	4.64	*
		1	2	3	4	5	6	7

Table 4.3 Comparisons between Genotype means

Mean vs Mean	Approx.t	Significant at p<0.05
ARQ/ARQ AHQ/ARQ	-0.771	No
ARQ/ARQ ARQ/ARR	-1.373	No
ARQ/ARQ ARR/ARR	-1.722	No
ARQ/ARQ AHQ/ARR	-2.017	No
ARQ/ARQ VRQ/VRQ	-2.691	Yes (p<0.05)
VRQ/ARQ AHQ/ARQ	-0.556	No
VRQ/ARQ ARQ/ARR	-0.905	No
VRQ/ARQ ARR/ARR	-1.321	No
VRQ/ARQ AHQ/ARR	-1.645	No
VRQ/ARQ VRQ/VRQ	-2.803	Yes (p<0.01)
AHQ/ARQ ARQ/ARR	-0.326	No
AHQ/ARQ ARR/ARR	-0.841	No
AHQ/ARQ AHQ/ARR	-1.047	No
AHQ/ARQ VRQ/VRQ	-1.285	No
ARQ/ARR ARR/ARR	-0.627	No
ARQ/ARR AHQ/ARR	-0.864	No
ARQ/ARR VRQ/VRQ	-1.142	No
ARR/ARR AHQ/ARR	-0.293	No
ARR/ARR VRQ/VRQ	-0.626	No
AHQ/ARR VRQ/VRQ	-0395	No

The relationship between C1 and incubation period
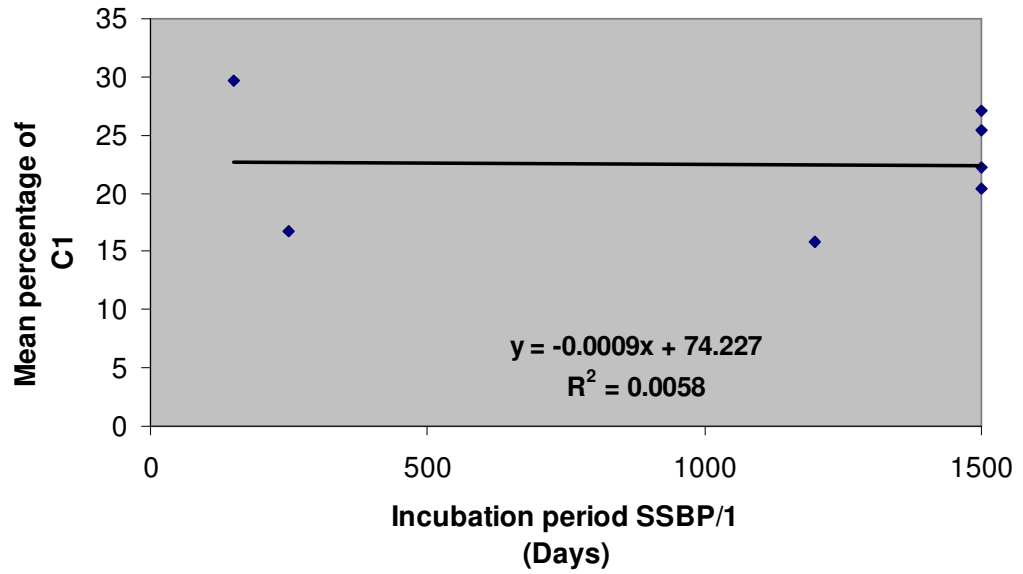
Figure 4.6 Correlation graph between mean percentage of C1 and incubation period with SSBP/1

Mean percentage of C1, for each genotype group measured by densitometry and the incubation period for each genotype after incubation with SSBP/1. Each point represents one genotype group. This graph clearly shows that there is no correlation between the amount of C1 and incubation period.

4.2.3 Full-length PrP and C1 percentages in young animals (<1 year)

As part of the genotype study, it was noted that three still born lambs had a higher percentage of C1 compared to adult sheep. To follow up this observation sheep with mixed genotypes from three different breeds (Cheviot, Poll Dorset and Suffolk) were divided into three age groups:

I) 0-5 day old, n = 6,

II) 100-320 day old, n = 5,

III) adult sheep one year or older, n = 44

The results for group (III) are presented under 4.2.1. The frontal cortex was homogenised and Western blotting was performed with antibody 6H4. Statistics was carried out using REML. After statistical analysis there was no significant difference between the age of the animal and the percentage of C1 ($p>0.05$).

However, the percentage of C1 in younger animals (0-5 day olds) was very low (Figure 4.7). In contrast animals in the 100-320 age group had much higher percentages of C1 which were comparable to animals in the one year and older age group (Figure 4.7 and Figure 4.8). Mean C1 percentage values for young animals are located in Table 4.4.

Table 4.4 Densitometry data for young sheep (Frontal cortex)

Genotype	Animal no	Age (days)	C1: total PrP^C Result 1	C1: total PrP^C Result 2
ARQ/ARQ	6D	0-5	5:95	2:98
ARQ/ARQ	2006E	0-5	15:85	7:93
ARQ/ARQ	2016E	0-5	12:88	19:81
AHQ/ARQ	82 x 15	0-5	14:86	11:89
AHQ/ARR	81 x 80	0-5	9:91	5:95
ARR/ARR	81 x 34	0-5	12:88	7:93
ARQ/ARQ	97A	100-320	30:70	27:73
ARQ/ARR	005D	100-320	5:95	3:97
ARQ/ARR	021D	100-320	37:63	32:68
ARQ/ARR	024D	100-320	21:79	21:79
ARQ/ARR	013D	100-320	37:63	22:78

Table showing densitometry data for young sheep. C1 is calculated as a ratio of total PrP^C and shown in the format C1: total PrP^C. Each sample was tested twice (shown as result 1 and result 2).
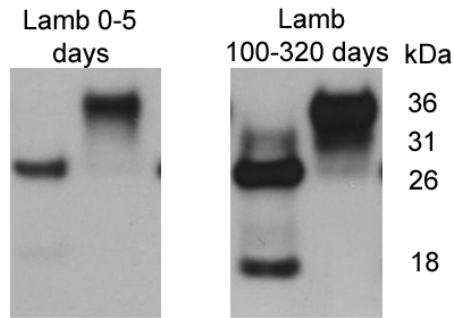
Figure 4.7 Representative Western blots of alpha cleavage profiles for lambs 0-5 days and lambs 100-320 days (Cortex) detected using monoclonal antibody 6H4

Western blot of PNGase deglycosylated and undigested PrP^C from lamb brain homogenate. Diglycosylated PrP^C detected at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 µl of 10% brain homogenate was loaded for each sample.
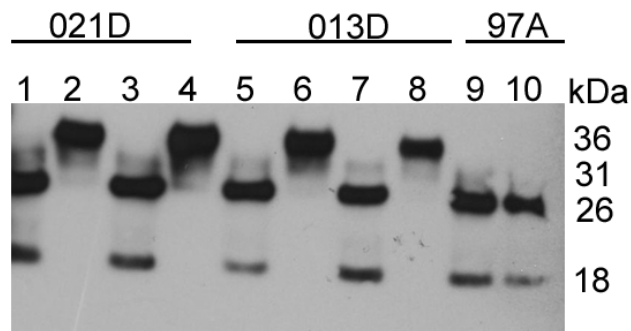
Figure 4.8 PrP and C1 detected from frontal cortex of three Suffolk lambs animals aged 100-320 days using monoclonal 6H4

Western blot of PNGase deglycosylated and undigested PrP^C from Suffolk sheep no. 021D, no. 013D and no. 97A. These animals are 100-320 days old. The diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 µl of 10% brain homogenate was loaded for each sample. This experiment was repeated twice (n = 2).

4.2.4 Full-length PrP and C1 in ovine brain regions

PrP was prepared from medulla, thalamus and cerebellum to compare the percentage of C1 in these brain regions to the previously established values for frontal cortex. The medulla was chosen as this area is one of the first to accumulate PrP^{Sc} deposition during scrapie disease (Jeffrey *et al*., 2001). The thalamus and cerebellum are also targeted during scrapie pathogenesis and also chosen because these brain areas had previously been analysed (Kuczius *et al*., 2007a).

Brain tissue was sectioned from six animals that were one year or older (Table 4.5). There was no scope for investigating the effect of breed, sex, genotype or age on differences between brain areas as the 11 animals come from 10 different breed/sex/genotype/age combinations. Brain areas were compared on the assumption that breed/sex/genotype/age do not affect brain area differences. Repeat observations on the same animal/brain area was averaged to give at most one measure per animal per brain area and a non-parametric sign test used as many observation are at the 0% limit.

After statistical analysis using a non-parametric sign test there was a significant difference in the percentage of C1 between the cortx and medulla ($p<0.05$) and thalamus and medulla ($p<0.05$) (Figure 4.12). Overall, the highest amount of C1 was found in the frontal cortex and thalamus and less so in the medulla and cerebellum (Figure 4.9 A and B, Figure 4.11, Figure 4.12).

The variance was calculated for each individual animal for each brain region (Data not shown). A pooled estimate of variance was also calculated for each brain region (All animals). For the cerebellum the pooled estimate of variance was 4.4 with a standard deviation of 2.10. For the medulla the pooled estimate of variance was 3.6 with a standard deviation of 1.90. For the cortex the pooled estimate of variance was 10.17 with a standard deviation of 3.18. Finally the pooled estimate of variance for the thalamus was 10.35 with a standard deviation of 3.21. The Variance for the medulla and cerebellum was low as many variances were 0.

The percentage of C1 in these four brain regions were also analysed for newborn lambs (0-5 days old) and young lambs (100-320 days). Western blot results from the newborn lambs (0-5 days old) show very low percentages of C1 in the medulla, cerebellum and thalamus (Figure 4.10) which is comparable to the results that were seen in the frontal cortex using 6H4 (Figure 4.7, Figure 4.10). For two young lambs from the age group 100-320 days low percentages of C1 was observed in the medulla but higher percentages were observed in the thalamus (data not shown). Mean C1 percentage values for animals used in the brain region study are located in Table 4.1 and Table 4.5.
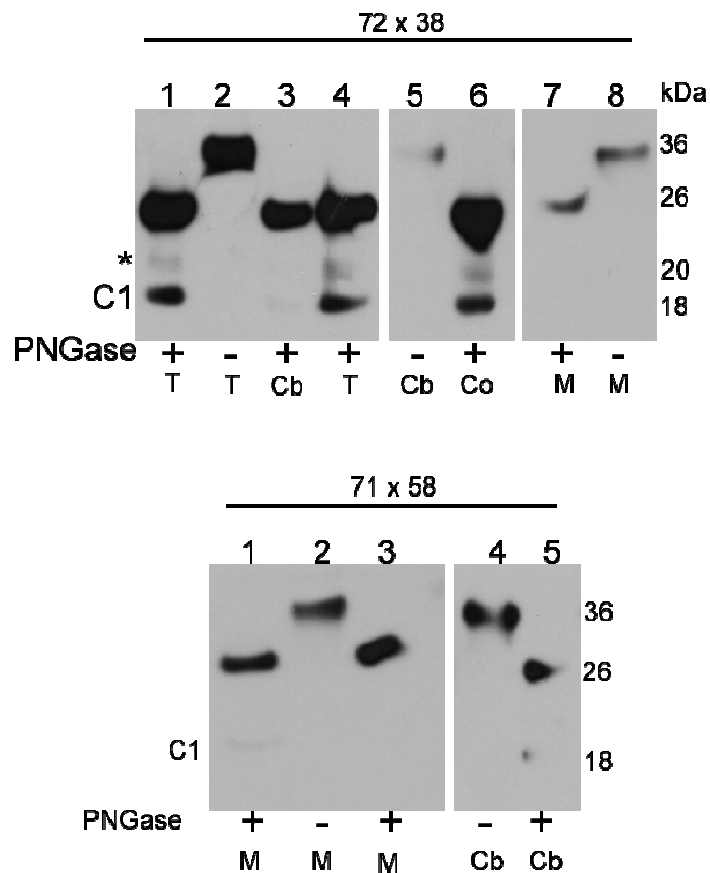
Figure 4.9 PrP^C and C1 detected in four areas of the sheep brain using monoclonal antibody 6H4

(A) Western blot profile of PNGase deglycosylated and undigested PrP^C from Cheviot sheep no. 72 x 38. High percentage of full-length PrP and C1 is detectable within the thalamus (T), (lane 1, 2 and 4) and the cortex (C) (lane 6). Only full-length PrP^C is detectable in the cerebellum (Cb), (lane 3) and medulla (M), (lane 7). There is also a band above C1 (most likely to be C2) (lane 1, 4 and 6) indicated by * on the blot at 20 kDa. (B) Western blot profile of PNGase deglycosylated and undigested PrP^C from Cheviot sheep no. 71 x 58. Only a small percentage of C1 is detected in the medulla (M), (lane 1) and cerebellum (Cb), (lane 5) of this animal. The diglycosylated form of PrP^C is detected at ~ 36 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. The monoglycosylated form of PrP^C is not detected in any of the undigested samples. 5 µl of 10% brain homogenate was loaded for each sample. Both experiments were repeated twice (n = 2).
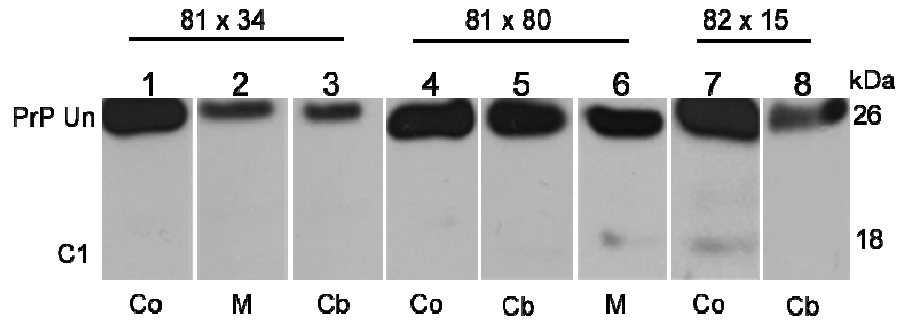
Figure 4.10 Western blots of alpha cleavage profiles from various ovine brain areas from newborn lambs (0-5 days old) detected using monoclonal antibody 6H4 (montage of different gels)

Western blot profiles of PNGase deglycosylated PrP^C from Cheviot sheep no. 81 x 34, 81 x 80, and 82 x 15. For sheep no. 81 x 34 only full-length PrP^C is detected in the cortex (Co), medulla (M) and cerebellum (Cb), (lanes 1-3). For animal no. 81 x 80 only full-length PrP^C is detected in the cortex (Co) and cerebellum (Cb), (lane 4 and 5). Full-length PrP^C and some C1 detected are detected in the medulla (M) of animal 81 x 80, (lane 6). For animal no. 82 x 15 full-length PrP^C and C1 are detected in the cortex (Co) (lane 7). Full-length PrP^C only is detected in the cerebellum (Cb) (lane 8). The unglycosylated form of PrP^C is detected at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 μl of 10% brain homogenate was loaded for each sample.
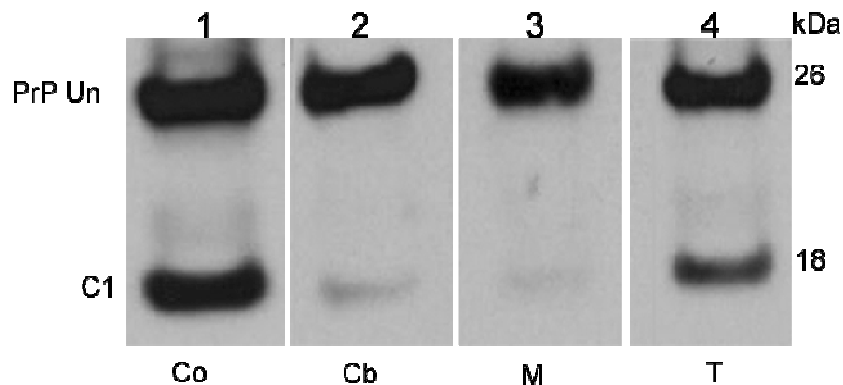
Figure 4.11 Detection of full-length PrP^C and C1 in different brain regions using monoclaonal antibody 6H4

Representative Western blot of alpha cleavage profiles from VRQ/VRQ homozygote animal J255. Full-length PrP^C and C1 were detected in the frontal cortex (Co), (Lane 1), cerebellum (Cb), (Lane 2), medulla (M), (Lane 3) and thalamus, (T) (Lane 4). The unglycosylated form of PrP^C is detected at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 μl of 10% brain homogenate was loaded for each sample.

Table 4.5 Densitometry data for brain region study (Excluding frontal cortex)

Genotype	Animal No	Age months	Brain area	C1:total PrP^C result 1	C1: total PrP^C result 2	C1: total PrP^C result 3
VRQ/VRQ	J255	12	Cerebellum	18:82	9:91	7:93
			Medulla	0:100	0:100	0:100
			Thalamus	36:64	34:66	29:71
ARQ/ARQ	69 x 67	84	Cerebellum	8:92	0:100	0:100
			Medulla	0:100	0:100	0:100
			Thalamus	0:100	0:100	0:100
VRQ/ARQ	H30	24	Cerebellum	0:100	0:100	0:100
			Medulla	0:100	0:100	0:100
			Thalamus	10:90	0:100	0:100
AHQ/ARQ	82 x 15	0	Cerebellum	0:100	0:100	0:100
AHQ/ARR	71 x 58	72	Cerebellum	0:100	0:100	0:100
			Medulla	0:100	0:100	0:100
			Thalamus	46:54	45:55	35:65
AHQ/ARR	81 x 80	0	Cerebellum	0;100	0:100	0:100
			Medulla	0:100	0:100	0:100
ARQ/ARR	013D	3-6	Medulla	0:100	0:100	-
			Thalamus	24: 76	20:80	-
ARQ/ARR	021D	3-6	Medulla	6:94	0:100	-
			Thalamus	23:77	15:85	-
ARQ/ARR	72 x 38	72	Cerebellum	0:100	0:100	0:100
			Medulla	0:100	0:100	0:100
			Thalamus	40:60	36:64	34:66
ARR/ARR	75 x 49	60	Cerebellum	0:100	0:100	-
			Medulla	5:95	0:100	-
			Thalamus	34:66	32:68	-
ARR/ARR	81 x 34	0	Cerebellum	20:80	18:82	16:84
			Medulla	10:90	0:100	0:100

Table showing densitometry data for brain region study. C1 is calculated as a ratio of total PrP^C and shown in the format C1: total PrP^C for each brain region tested. Each sample was tested twice or three times (shown as result 1, result 2 and result 3).
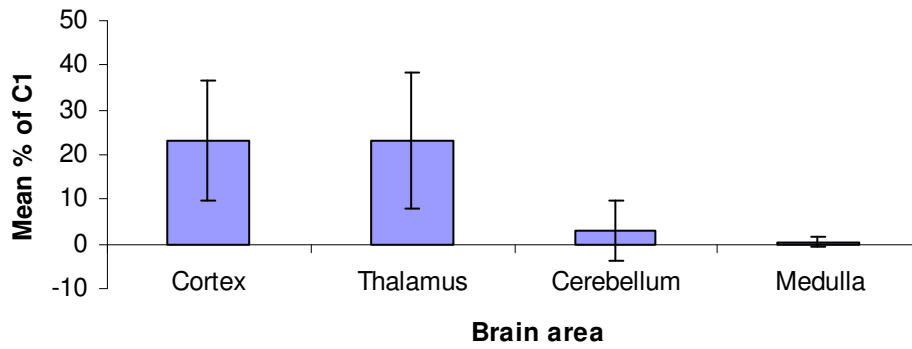
Figure 4.12 Histogram of mean C1 % values for all ovine brain regions

Error bars indicate the standard deviation for animals for each brain area. After analysis using non parametric sign test the mean amount of C1 in the frontal cortex and the mean amount of C1 in the thalamus is significantly different to that in the medulla ($p < 0.05$).

4.2.5 Quantifying the amount of PrP^C in four different regions of the sheep brain

As PrP^C expression is important for the development of TSE disease, the amount of PrP^C in different areas of the sheep brain was quantified using the enhanced lanthanide fluoroimmunoassay (DELFIA®) with a previously established protocol. Five animals that had been used previously for protein detection by Western blot analysis were used for this quantitative ELISA. The DELFIA® technique uses pairs of PrP specific antibodies to measure the amount of PrP in a sample. In this experiment capture antibody FH11 (epitope 54-57, Figure 3.11) which will only capture full-length PrP^C was used. The DELFIA® assay conditions were not established to use antibodies that would detect C1 and full-length PrP. Antibody 8H4 (epitope 175-185, Figure 3.11) labelled with lanthanide europium was used to detect bound PrP. For this assay the amount of PrP^C in an individual sample was determined using sheep recombinant PrP (ARQ allele) as a standard and assay buffer as a negative control.

To confirm that these animals were uninfected, PrP^C was solubilised in 2M guanidine hydrochloride in which PrP^{Sc} is insoluble. After initial treatment with guanidine hydrochloride a pellet remained. This pellet was solubilised in 6M guanidine hydrochloride. PrP^{Sc} is soluble at this concentration and would be detectable if it was present. Total protein concentrations were carried out for each sample (2.1.3) before treatment with guanidine hydrochloride. All the samples that were used in the DELFIA® assay were negative for PrP^{Sc} (Data not shown). For these experiments, the cortex, medulla, cerebellum and thalamus from five Cheviot sheep with mixed genotypes were analysed, each sample was assayed twice in duplicate.

Statistical analysis was not performed because only five animals were used in this study. The results for all sheep tested are presented as histograms in Figure 4.13. Total PrP values for these animals take into account the C1 fragment by adding the mean percentage of C1 for each individual brain area using densitometry measurements from the Western blot results of each animal.
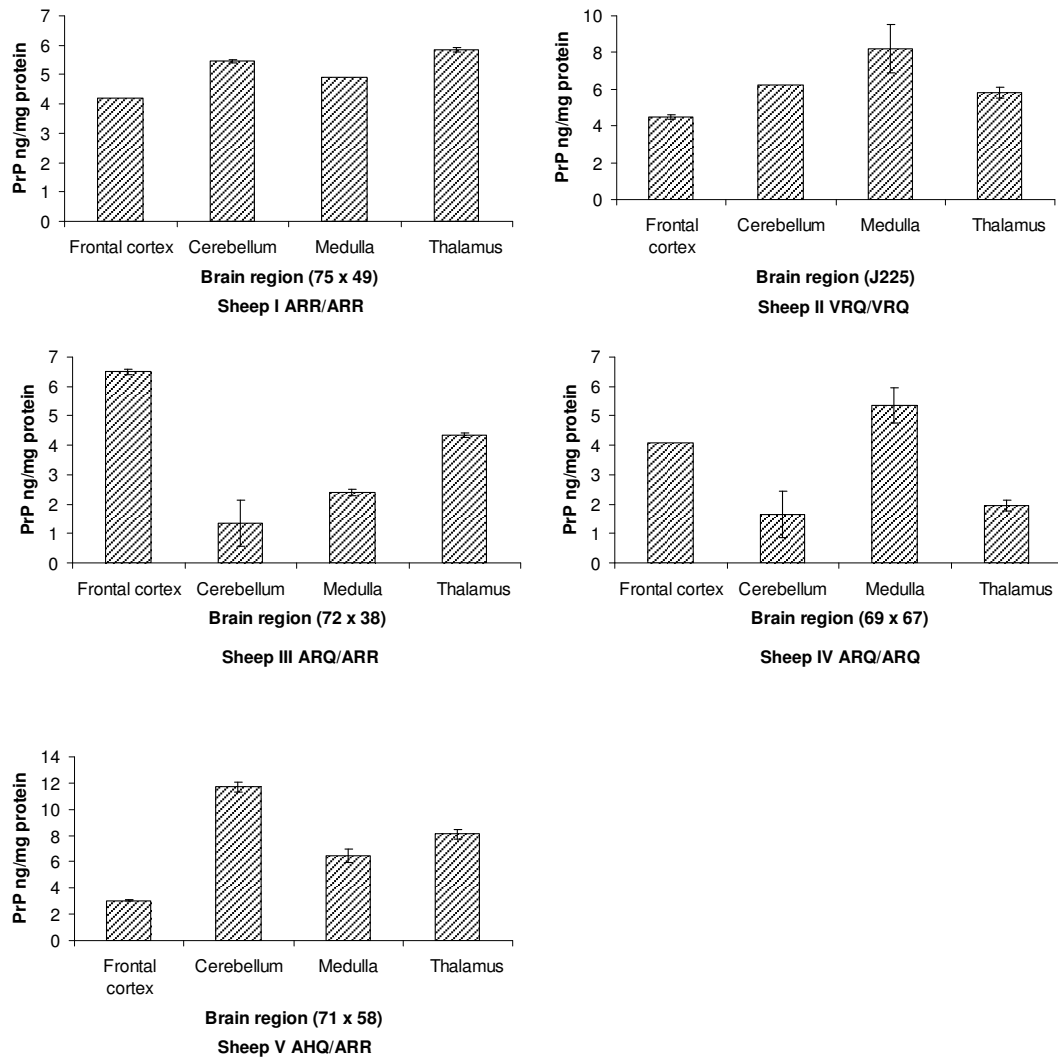
Figure 4.13 Mean amount of soluble PrP^C (Including C1) detected by DELFIA$^®$ and Western blot for four different regions of the ovine brain for individual animals

PrP^C quantification in different brain regions is expressed in ng/mg of total protein for each tissue. Full-length PrP^C was measured using the DEFLIA$^®$ and this was added to the mean amount of C1 which was measured from the Western blot results to get an estimate total PrP^C value (Full-length PrP^C + (Full-length PrP^C x % C1) = Total PrP^C. Each sample was assayed in duplicate and the corresponding means calculated. Error bars indicate the standard deviation between repeats for each brain region for the DELFIA assay. In cases where no error bar is shown, exact replicate values were produced. Each sample was repeated twice n = 2.

The overall distribution of PrP^C for all sheep was variable between each animal and between individual brain areas of the same animal (Figure 4.13). The least variation was observed for animal 75 x 49 with mean values of 4.1 ng/mg for the cortex, 4.9 ng/mg for the medulla, 5.5ng/mg for cerebellum and 5.9 ng/mg for the thalamus. The most variation was observed for animal 71 x 58 with mean values of 3.1 ng/mg for the cortex, 6.5 ng/mg for the medulla, 11.7 ng/mg for the cerebellum and 8.1 ng/mg for the thalamus.

For sheep 75 x 49 the highest amount of PrP was detected in the thalamus and lowest in the cortex. For sheep J225 the highest amount of PrP was detected in the medulla and lowest in the cortex. For sheep 72 x 38 the highest amount of PrP was detected in the cortex and lowest in the cerebellum. For sheep 69 x 67 the highest amount of PrP was detected in the medulla and the lowest in the cerebellum. And finally for sheep 71 x 58 the highest amount of PrP was detected in the cerebellum and lowest in the cortex. These results are very varied and are different to the Western blot results although the Western blot results were not quantitative (Table 4.1 and Table 4.5). Results from the Western blots show strong PrP signals in the cortex and thalamus and lower signals for PrP in the cerebellum and medulla. The DELFIA$^®$ results are opposite for some of the sheep tested with higher amounts of PrP detected in the medulla and cerebellum and lower amounts of PrP in the cortex.

4.3 Discussion

4.3.1 The association of PrP^C alpha cleavage and *PRNP* genotype

The presented analysis of 44 adult sheep shows that PrP alpha cleavage occurs in all seven genotypes that were tested. It appears that the VRQ homozygote animals have a significantly higher percentage of C1 than ARQ/ARQ and VRQ/ARQ, genotypes, under the premise that breed and sex have, if at all, only a minor effect on this biochemical process and that only animals over three months of age are compared (REML analysis). The effect of breed was tested as the VRQ/VRQ group contained both Cheviot and Poll Dorset animals, however, no statistical difference was

observed between these groups although, this may be due to the small number of animals in each group n= 5. The sex ratio of the animals used in the genotype study is almost 1:3 male to female with some female only genotype groups. It is highly unlikely that sex has an effect on alpha cleavage as no differences in PrP between the sexes in any species have been published. In this study the amount of C1 that was detected in young lambs (0-5 days old) occurred at very low and often immeasurable levels, however these measurements were not significantly different to the amount of C1 detected in adult sheep. This data is supported by Laffont-Proust *et al*., (2005) who show no correlation between age and the amount of C1 in human brain samples. Our results may have been influenced by the low sample size of the young lambs (n = 5).

Although the REML analysis showed that there was a statistical significant difference between the amount of C1 between VRQ/VRQ homozygotes and ARQ/ARQ homozygotes and between VRQ/VRQ homozygotes and VRQ/ARQ heterozygotes, there is no correlation between the amount of C1 and incubation period with SSPB/1 scrapie (Figure 4.6). There was also no significant difference in the amount of C1 between the VRQ/VRQ homozygotes and the ARR/ARR homozygotes, which is the opposite end of the susceptibility range. This suggests the alpha cleavage does not play a major role in disease susceptibility.

Measuring the percentage of total PrP^C and C1 by Western blot has been shown to be reproducible. However the issue of enzymatic cleavage needs further investigation. In many cases before deglycosylation only diglycosylated PrP^C was detectable and after deglycosylation the C1 was visible. It was previously thought that deglycosylation was required for the visualisation of the C1 fragment as the C1 fragment migrates at the same size as unglycoyslated PrP^C only becoming visible after removal of the sugars. It may be that the enzyme PNGase F, which is used for the deglycosylation of PrP^C may be responsible for this cleavage, however this is unlikely as in some samples C1 was not always detectable after PNGase treatment. It may be that during PrP^C digestion with PNGase, some of PrP^C is lost during this process but this again seems unlikely as experiments in this thesis have shown that

PrP^C degradation does not occur after freeze thawing or after incubation of tissue at RT for 16 hours. It may be that degradation occurs more quickly with homogenates rather than fresh tissue.

By contrast a DELFIA/ELISA based approach provides quantitative analysis but requires more optimisation and a minimum of three different antibodies would have been necessary to get the same end result as it has been shown that antibody characteristics can significantly influence the outcome (Frances Wiseman, personal communication). DELFIA/ELISA based assays would also not have differentiated easily between C1, and C2 or potentially other fragments which is a great advantage of the Western blot as shown in several examples in this thesis.

The sequence of the PrP hydrophobic region around amino acid codons 106 –119 was identical for all animals that were used in the genotype study. This suggests that differences in the amount of cleavage can not be explained by the PrP hydrophobic sequence. Oliveira-Martins *et al*., (2010) show that only large deletions within the 106-119 domain of PrP impair the production of the C1 fragment substantially supporting the idea that the sequence although not necessarily the amino acid composition of the hydrophobic region is not essential for alpha cleavage. However, PrP primary sequence has been shown to be important for PrP alpha cleavage as human PrP^C showed an increase in C1 levels compared to mouse PrP^C in an *in vitro* study (Haigh *et al*., 2009).

Overall our results indicate that PrP alpha cleavage either has no biological consequence on disease or incubation period or that this association with disease is much more subtle and may only explain specific agent-host combinations. More experimental data will be needed to clarify this issue.

4.3.2 Comparisons with other studies of PrP^C cleavage ratios

Kuczius *et al*., (2007a) showed that the signal intensity of the C1 fragment was higher compared to full-length PrP^C in pooled and single sheep brain tissue samples

(Table 4.4). Our results differ fundamentally from Kuczius et $al.$, (2007a) with full-length PrP^C being dominant over the C1 fragment (Table 4.6) in all examined tissues. A direct comparison is difficult as different antibodies were used between the two studies. One might argue that the differences may be attributed to the use of different antibodies with their different epitopes as Kuczius et $al.$, (2007a) showed differences with various antibodies in their study. Alternatively, their procedure or storage of samples may be more prone to preferential degradation of PrP^C. It is also not apparent from their study what genotypes were tested, which may be very different from the ones tested here.

4.3.3 PrP^C fragments in relation to brain region

Previous work using RT-PCR and Western blotting show that expression levels of PrP in sheep brain are highest in the frontal cortex and lower in the obex and thalamus (Tichopad et $al.$, 2003, Diaz-San Segundo et $al.$, 2006, Gossner et $al.$, 2009) although a study by Han et $al.$, (2006) shows that PrP expression is highest in the obex.

In our study we quantified PrP expression levels in four regions of the sheep brain using the DELFIA$^®$ assay. Expression of PrP in certain brain areas may be correlated with the accumulation of PrP^{Sc} and the onset of disease. In our study we were limited to only five animals with varying $PRNP$ genotypes. The DELFIA$^®$ results suggest that there is some within and between animal differences between the amounts of PrP^C in the different brain areas. These differences may be due to varying expression levels of the enzyme responsible for the cleavage in these different brain areas. The results for the Western blot data and DELFIA$^®$ do not correlate well together, however these differences may be due to differences in antibodies that were used in both assays. As an internal control ovine ARQ/ARQ recombinant PrP was used to produce a standard curve, in which all our samples fell within the linear range.

When the results are compared to data by others the amount of PrP^C detected in our animals are within the same range as results by Moudjou et $al.$, (2001). In the study by Moudjou et $al.$, (2001) the amount of PrP detected was between 3-5 ng/mg of

total protein whereas the amount of PrP detected in our quantification was between 2-12 ng/mg of total protein. Differences observed between our results may be due to the differences between the DELFIA® assay and the standard ELISA methods used by Moudjou *et al.*, (2001) although similar principles apply. The antibodies used in our DELFIA® was also different to the antibodies used by Moudjou *et al.*, (2001). It is known from previous studies that the pairing of antibodies in the DEFLIA assay can affect results (Frances Wiseman, personal communication).

Finally it is unknown whether alpha cleavage of the prion protein is involved in conversion of PrP^C to PrP^{Sc}. After analysing the percentage of C1 in animals with varying degrees of susceptibility to scrapie it seems unlikely that the amount of C1 is directly involved in scrapie disease.

Table 4.6 Dominant PrP forms detected in sheep brain tissue

Study	Antibodies	Brain region	Dominant PrP form
Kuczius *et al.*, 2007a	SAF70 SAF34 Pri308 12F10	Pooled	C1
	SAF70	Cortex	C1
		Cerebellum	
		Brain stem	
Love *et al.*, 2009 data presented in this thesis	6H4	Cortex	Full-length PrP^C
		Thalamus	
		Medulla	
		Cerebellum	

Chapter 5 Alpha cleavage in PrP transgenic mouse models

5.1 Introduction

PrP transgenic mice have been used extensively in prion research to address questions about pathology (Bueler *et al*., 1992, Manson *et al*., 1994, Moore *et al*., 1995) transmission (Bruce *et al*., 1994, Scott *et al*., 1999), PrP glycosylation (Cancellotti *et al*., 2005) and about the normal function of PrP (Sakaguchi *et al*., 1996, Moore *et al*., 1999). As yet transgenic mice have not been used to study PrP alpha cleavage.

As shown in chapter 4, alpha cleavage can occur at a relative high level of almost 50% of total PrP in sheep cortex. Two different transgenic models were available to investigate the relationship between the amount of PrP that is produced in a tissue, the sequence of PrP and the level of PrP alpha cleavage. The first model is represented by highly over-expressing transgenic mice Tg20, Tg338 and KB6. The second model represents moderately over-expressing transgenic mice with different PrP protein variants, such as KB5, KVRQ and KARQ.

Tg20 mice express murine PrP at ten-times the level of wild type mice. These mice are very susceptible to mouse scrapie and have an incubation period of ~ 63 days compared to 149 days for wild type mice (Brandner *et al*., 1996, Fischer *et al*., 1996). Tg338 mice are transgenic for the VRQ allele of ovine PrP on a mouse PrP$^{0/0}$ background. The PrP expression levels in the brains of Tg338 mice are 8-to-10 fold that in sheep (Le Dur *et al*., 2005). These mice are also susceptible to scrapie and have short incubation times compared to sheep with the same genotype (Vilotte *et al*., 2001, Le Dur *et al*., 2005, Thackray *et al*., 2008). Line KB6 expresses a bovine PrP variant with six octapeptide repeats (Goldmann *et al*., 1991b) at about 6 times the level of normal mouse brain. KB5 mice express a bovine PrP variant with five octapeptide repeats at twice the level of normal mouse brain.

Lines KVRQ and KARQ were chosen as these two models express ovine VRQ and ARQ alleles respectively, at 1-2x level of normal brain. The last four mouse lines were derived from founders provided by Prof G. Telling, University of Kentucky. All four lines are on mouse PrP$^{0/0}$ background. Unpublished data for the KARQ and KVRQ transgenic mice by Hunter and colleagues (Roslin Institute) show that the relationship between incubation period and PrP genotype are equivalent to what has been described for sheep (Goldmann *et al*., 1994, Houston *et al*., 2002a). This suggests that these mice are a good scrapie disease model, but it remains to be seen whether they are a good model for PrP^C biology, e.g. alpha cleavage. 129/Ola mice that express murine PrP at normal level were used as base line control.

Another very different PrP-transgenic model that was used to investigate principles of PrP^C cell biology was mice deficient in PrP glycosylation. These mice are gene targeted and carry mutations at the first, second or both Asn-linked glycosylation sites of PrP. They are referred to as G1 (codon 180 Thr), G2 (codon 196 Thr) and G3 (codon 180 Thr + codon 196 Thr) mice (Cancellotti *et al*., 2005, Tuzi *et al*., 2008). The substitution of asparagine with threonine at position 180 in the G1 mice and at position 196 in the G2 mice will only allow the production of mono- and unglycosylated PrP. G3 mice with the threonine substitution at positions 180 and 196 only produce unglycosylated forms of PrP (Cancellotti *et al*., 2005, Tuzi *et al*., 2008).

In G1 and G2 mice, PrP is localised at the cell membrane (Cancellotti *et al*., 2007) and in G3 mice it is found mainly within the golgi apparatus (Cancellotti *et al*., 2007). These models therefore provide a means of investigating cellular pathways and the role of glycosylation on alpha cleavage of PrP.

This chapter analyses whether cleavage can occur in various transgenic models with varying levels of PrP and whether alpha cleavage occurs in PrP glycosylation deficient mice.

5.2. Results

5.2.1 Detection of C1 in ovine PrP expressing transgenic mice

Brain homogenates from transgenic mice from lines KVRQ and KARQ, expressing ovine PrP variants VRQ and ARQ, respectively were analysed by Western blotting and C1 as a percentage of total PrP^C was measured by densitometry. Presence of the VRQ and ARQ alleles were confirmed by direct sequencing of PCR products (2.2.7). Figure 5.1 (A and B) show C1 cleavage profiles from two KARQ and two KVRQ transgenic mice.
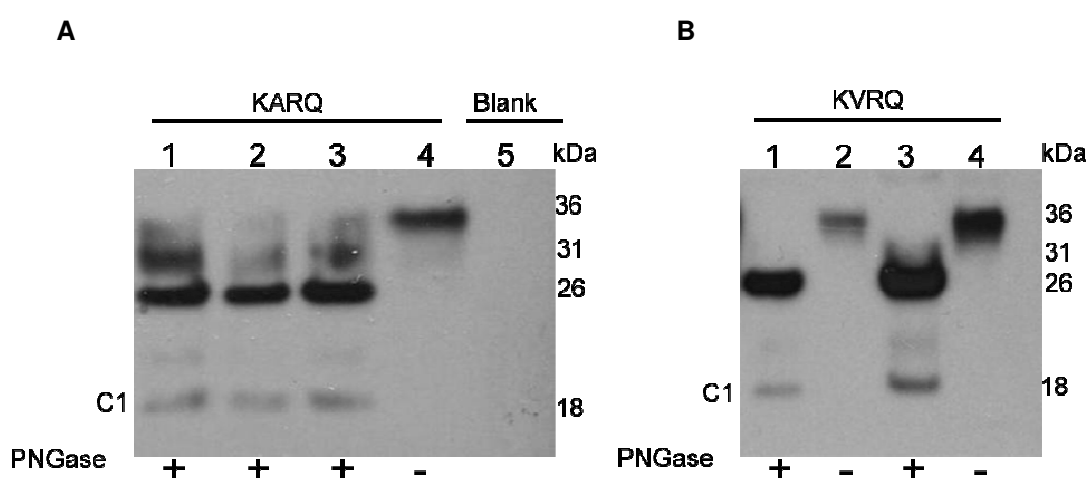
Figure 5.1 PrP and C1 detected from KARQ and KVRQ transgenic mice using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C of two KARQ transgenic mice. PrP^C is mostly diglycosylated (lane 4), control buffer only (lane 5). C1 is present in all PNGase treated samples (Lane 1, 2 and 3). Animal 1 (lane 1, 3 and 4), animal 2 (lane 2). Some remaining mono and diglycosylated PrP^C can be seen in lanes 1-3. (B) Western blot of PNGase deglycosylated and undigested PrP^C of two KVRQ transgenic mice. Animal 1 (lane 1 and 2), animal 2 (lane 3 and 4). PrP^C is most diglycosylated. C1 is present in both PNGase treated samples. The diglycosylated form of PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. 5 µl of 10% brain homogenate was loaded for each sample. Each experiment was repeated twice (n = 2).

Tg338 mice were analysed by Western blot and C1 as a percentage of total PrP^C was measured by densitometry. Cleavage profiles from Tg338 mouse brain homogenates with antibody 6H4 are shown in Figure 5.2A. This profile proves that alpha cleavage does occur in these transgenic mice but at much higher percentage than in KVRQ and KARQ mice (Figure 5.1A and B). When 20µg of total protein is loaded (Figure 5.2A, lane 2) there is also another truncated fragment at approximately 20kDa that is visible in substantial amounts. This fragment could be glycosylated C1, the C2 fragment or a mixture of both. However, when the amount of total protein from the Tg338 homogenate was reduced from 20µg/ml to 10µg/ml to achieve complete PNGase digestion the amount of the 20kDa band is reduced compared to the previous preparation (Figure 5.2A, lane 4, 6). As expected C1 as a percentage of total PrP^C does not change when 10µg of protein is loaded and all glycosylation has been removed from PrP (Figure 5.2A, lane 4, 6). Using antibody P4 that will recognise C2 but not C1, two bands are detected suggesting that they are full-length PrP^C and the C2 fragment, respectively (Figure 5.2B).

A

B
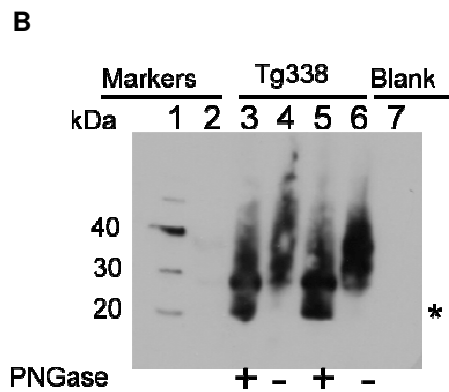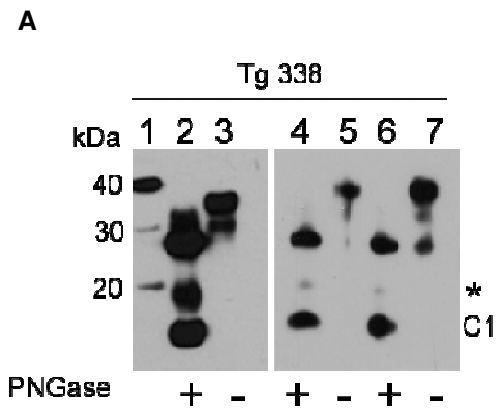
Figure 5.2 PrP and C1 detected from Tg338 mouse brain homogenate

(A) Western blot profile of Tg338 mouse brain homogenate treated (lane 2, 4, 6) and untreated (lane 3, 5, 7) with PNGase and detected with monoclonal antibody 6H4. Molecular weight marker loaded in lane 1. C1 is detected in PNGase positive samples along with an extra band (most likely to be C2) (indicated by *) at ~ 20kDa. 20 μg of total protein loaded (lane 2, 3) and 10 μg of total protein loaded (4, 5, 6, 7). (B) Western blot profile of Tg338 mouse brain homogenate detected using P4. Molecular weight marker loaded in lane 1. C2 fragment (*) which is detected at ~ 20 kDa is only detected in PNGase treated samples. 20 μg of total protein loaded for each sample. The diglycosylated form of PrP^C is detected at ~ 36 kDa, monoglycosylated PrP^C at ~ 32 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 17 kDa. Both experiments were repeated twice (n = 2).

In a serial dilution series Tg338 mouse brain homogenate was diluted 1:125 which resulted in a signal similar or slightly less than the level of a normal mouse brain at a 1:5 dilution (Figure 5.3, lanes 5 and 6). This suggests that there is a 15-25 fold difference in the levels of PrP between the over expressing mice and the non transgenic mice (Figure 5.3).
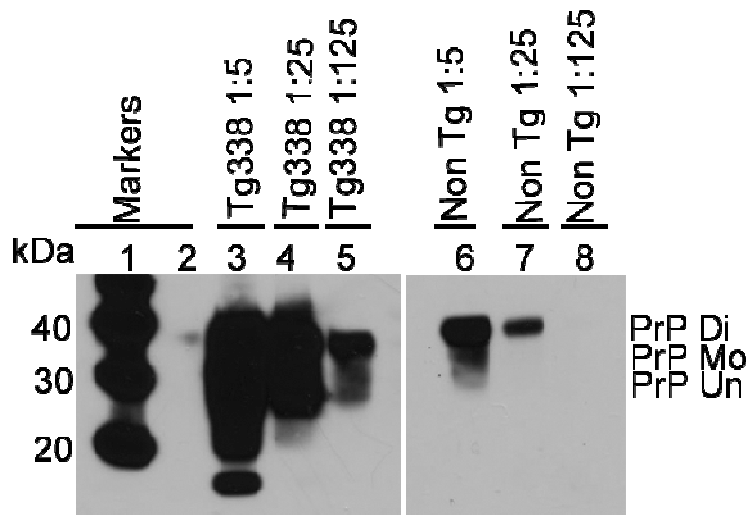
Figure 5.3 Dilution series of PrP^C in Tg338 and non-transgenic 129/Ola mouse brain homogenate using monoclonal antibody BC6

Western blot of PrP^C from frontal cortex homogenate (10%). The dilution values for the 10% brain homogenate from Tg338 and 129/Ola mice are indicated. Molecular weight marker loaded in lane 1. Colour standard loaded in lane 2. 20 µl of 10% brain homogenate was loaded for each sample. The diglycosylated form of PrP^C is detected at ~ 38 kDa, monoglycosylated PrP^C at ~ 32 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 fragment at ~ 18 kDa. This experiment was carried out twice (n = 2).

5.2.2 Detection of C1 in murine PrP expressing transgenic mice

Observations similar to the Tg338 mice were seen for murine PrP transgenic Tg20 mice which also over-express PrP. Figure 5.4 show C1 cleavage profiles from Tg20 and 129/Ola brain homogenates. Alpha cleavage does occur in these transgenic mouse models, with the percentage of C1 much greater in the Tg20 mice compared to the 129/Ola.

The ratio of total PrP^C/C1 produced in the 129/Ola mice (Figure 5.4, lane 1, 3) is equivalent to the ratios of total PrP^C/C1 produced in the KVRQ (Figure 5.1B, lane 1, 3) and KARQ (Figure 5.1A, lane 1, 2, 3) which also express approximately normal protein levels. The percentage of C1 produced in the Tg20 is similar to the percentage of C1 produced in the Tg338 mice (Table 5.1). An extra band of approximately 20kDa which is thought to be the C2 fragment is also seen in the Tg20 mice (Figure 5.4, lane 5, 6). This band is also observed in the over-expressing ovine PrP mouse line (Figure 5.2 A).

Densitometry data for transgenic mice (C1 expressed as a percentage of total PrP^C) for all ovine and murine PrP transgenic mice and 129/Ola mice can be found in Table 5.1. Densitometry measurements were also taken for the C2 band for Tg338, and Tg20 mice, Densitometry data for C1, C2 and Full-length PrP (FL PrP^C) can be found in Table 5.2. C1 was measured as a percentage of C2 and Full length PrP^C i.e (C1 /C1+C2+ Full length PrP^{C*} 100). For Tg338 and Tg20 mice the amount of C1 was always greater than the amount of C2 (Table 5.2).
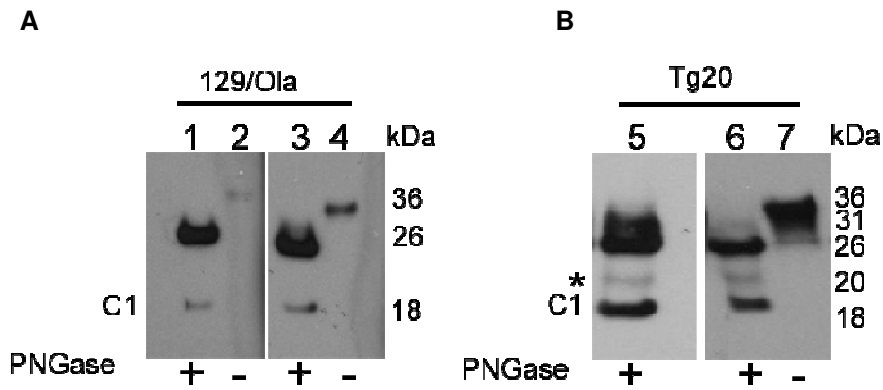
Figure 5.4 PrP^C and C1 detected from 129/Ola and Tg20 mice brain homogenates using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C of two 129/Ola mice. PrP^C is mostly diglycosylated (lane 2, 4). C1 is present in all PNGase samples (lane 1, 3). (B) Western blot of PNGase deglycosylated and undigested PrP^C of two Tg20 mice. C1 is present in all the PNGase samples (lane 5, 6). An extra band can be seen in the PNGase samples (most likely to be C2) (lane 5, 6) indicated by * on the blot. This band is at ~ 20 kDa. Some remaining mono-glycosylated PrP^C can be seen in lane 5, 6. Diglycosylated PrP^C is at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 and the C1 fragment at ~ 18 kDa. 5 μl of 10% brain homogenate was loaded for each sample. This experiment was repeated twice (n = 2).

5.2.3 Detection of C1 in bovine PrP expressing transgenic mice

Bovine PrP transgenic mice KB6 mice that over-express PrP with six octapeptide repeats and KB5 mice that express PrP at normal levels with five octapeptide repeats were analysed by Western blotting and C1 as a percentage of total PrP^C was measured by densitometry. C1 cleavage profiles from KB6 and KB5 mice are presented in Figure 5.5 (A and B). These profiles show that alpha cleavage does occur in these transgenic mouse models although the percentage of C1 is more in the KB6 mice compared to the KB5 mice (Figure 5.7).

The percentage of C1 in the KB6 mice is equivalent to the percentage of C1 found in the other over expressing transgenic mice Figure 5.6 (A, B and C). The percentage of C1 found in the KB5 mice is equivalent to other transgenic mice expressing normal levels of PrP and equivalent to wild type mice Figure 5.6 (A, B and C). The C2 band is also visible in the KB6 model. Representative Western blots for all transgenic mouse models are shown in Figure 5.6 (A, B and C). Densitometry data for transgenic mice (C1 expressed as a percentage of total PrP^C) for all KB5 and KB6 mice can be found in Table 5.1. Densitometry measurements were also taken for the C2 band for KB6 mice, Densitometry data for C1, C2 and Full-length PrP (FL PrP^C) can be found in Table 5.2. C1 was measured as a percentage of C2 and Full length PrP^C i.e (C1 /C1+C2+ Full length PrP$^{C\,*}$ 100). For KB6 mice the amount of C1 was always greater than the amount of C2 (Table 5.2).
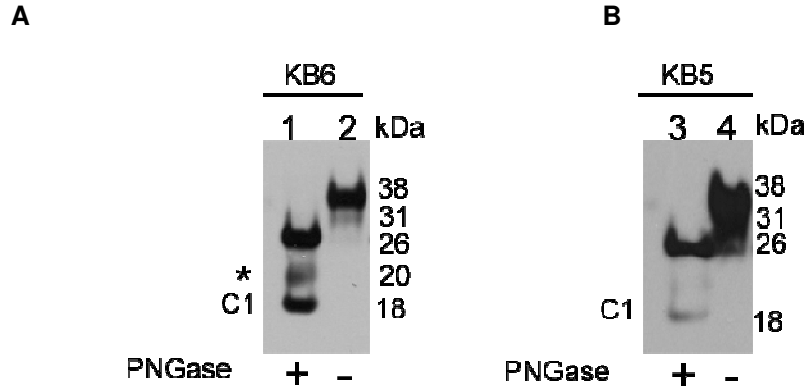
Figure 5.5 PrP^C and C1 detected from KB6 and KB5 mice brain homogenates using monoclonal antibody 6H4

(A) Western blot of PNGase deglycosylated and undigested PrP^C of one KB6 mouse. PrP^C is mostly diglycosylated. The C1 fragment is present in the PNGase sample (lane 1). An extra band can be seen in the PNGase sample (most likely to be C2) (lane 1) indicated by * on the blot. This band migrates at ~ 20 kDa. Some remaining monoglycosylated PrP^C can also be seen in lane 1. (B) Western blot of PNGase deglycosylated and undigested PrP^C of one KB5 mouse. The C1 fragment is present in the PNGase sample (lane 3). Diglycosylated PrP^C is at ~ 38 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa and the C1 at ~ 18 kDa. 5 µl of 10% brain homogenate was loaded for each sample. Both experiments were repeated twice (n = 2).
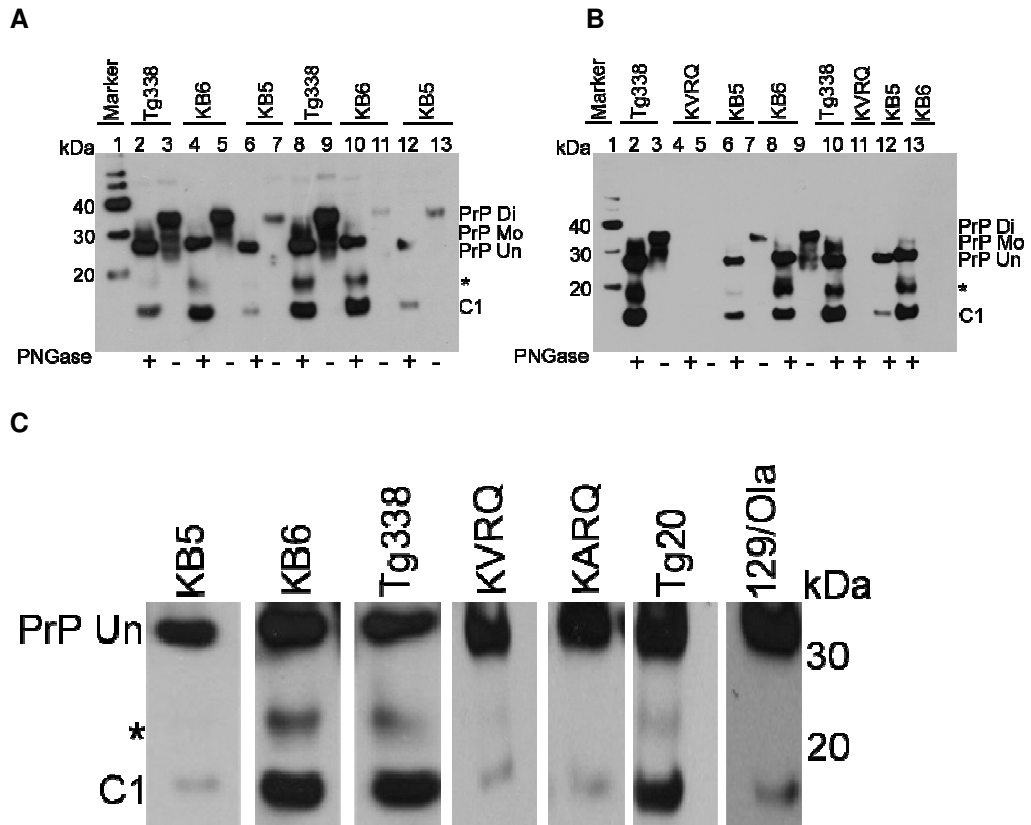
Figure 5.6 Representative Western blots of PrP and C1 from transgenic mouse brain homogenates using monoclonal antibody 6H4

(A, B and C) Western blot of deglycosylated PrP^C from mouse brain tissue. Protein bands were detected as full-length unglycosylated PrP^C (~30 kDa) and the C1 fragment at (~18 kDa) using antibody 6H4. An increase in levels of C1 was observed in the over expressing KB6, Tg338 and Tg20 mouse models. A band at ~ 20 kDa most likely to be the C2 fragment indicated by * on the blot was present in higher quantities in the over-expressing transgenic models. 5 μl of 10% brain homogenate was loaded for each sample.

Table 5.1 Densitometry data for transgenic mice

Transgenic model	Expression level	Tissue sample	C1 : (Full length PrP^C+C2) Result 1	C1 ; (Full length PrP^C+C2) Result 2
KVRQ	2x	Mouse 1	5:95	5:95
		Mouse 2	14:86	11:89
		Mouse 3	22:78	21:79
		Mouse 4	20:80	15:85
KARQ	2x	Mouse 1	11:89	10:90
		Mouse 2	21:79	18:82
		Mouse 3	15:85	14:86
		Mouse 4	13:87	12:88
KB5	2x	Mouse 1	11:89	7:93
		Mouse 2	9:91	7:93
KB6	6x	Mouse 1	34:66	30:70
		Mouse 2	35:65	34:66
Tg20	10x	Mouse 1	29:71	25:75
		Mouse 2	23:77	21:79
Tg338	8x	Mouse 1	43:57	31:69
		Mouse 2	27:73	25:75
129/Ola	1x	Mouse 1	9:91	8:92
		Mouse 2	10:90	10:90

Table showing densitometry data for all transgenic mice. C1 is calculated as a ratio of total PrP^C and shown in the format C1:C1 :**(Full length PrP^C+C2)** Each sample was tested twice (shown as result 1 and result 2). Western blots performed with 6H4.

Table 5.2 Densitometry data for transgenic mice (ratio of C1:C2:Full length PrP^C)

Transgenic model	Expression level	Tissue Sample	C1:C2:Full length PrP^C Result 1	C1:C2: Full length PrP^C Result 2	Expression level
KB6 1	6x	Mouse 1	34:27:39	30:12:58	6x
KB6 2	6x	Mouse 2	35:19:46	34:18:48	6x
Tg20 1	8x	Mouse 1	29:0:27	25:13:62	10x
Tg20 2	8x	Mouse 2	23:17:60	21:0:79	10x
Tg338 1	10x	Mouse 1	43:0:57	31:17:52	8x
Tg338 2	10x	Mouse 2	27:19:54	25:21:54	8x

Table showing densitometry data for all transgenic mice (ratio of **C1:C2:Full length PrP^C**).Each sample was tested twice (shown as result 1 and result 2). In some cases the C2 fragment was not present this is indicated by 0).Western blots performed with antibody 6H4.

5.2.4 Comparison between the amount of PrP^C in over expressing Tg338 mice and normal PrP expressing KVRQ and KARQ mice

To quantify how much prion protein is produced in PrP over-expressing mice, the amount of PrP^C in the brains of over-expressing ovine PrP transgenic Tg338 mice and normal expressing ovine PrP transgenic KARQ and KVRQ mice were compared by dissociation enhanced lanthanide fluorimmunoassay (DELFIA$^®$).

In this experiment capture antibody FH11, which will only capture full-length PrP, was used as before. Antibody (8H4) labelled with lanthanide europium was used to detect bound PrP. (For detailed methodology see section 2.3.4 and 2.1.11.4). For this assay the amount of PrP^C in an individual sample was determined using sheep recombinant PrP (ARQ allele) as a standard. Assay buffer was used as a negative control. 129/Ola mice could not be used as direct control because mouse recombinant PrP would be required as a standard to obtain accurate measurements. For these experiments each animal was assayed twice in duplicate.

The results for all transgenic mice tested are presented as a table 5.3. PrP values for these animals take into account the C1 fragment by adding the mean percentage of C1 for each individual transgenic line, using densitometry measurements from the Western blot results. The amount of protein loaded was not controlled as not enough tissue was available for this assay; therefore a total protein assay was not carried out. Instead 50 µl of a 10% w/v brain homogenate was loaded for each sample. The values are given as ng/ml as this corresponds to the value given for the standard curve.

Results from the DELFIA$^®$ indicate that Tg338 mice express approximately 10 x more PrP than KVRQ and KARQ mice, which is less than the calculated values for the Western blot quantification (Figure 5.3). The DELFIA$^®$ results for the KARQ and KVRQ mice were similar with a mean value of 0.7 ng/ml and 0.4 ng/ml, respectively. Because only two or three animals were used in this study per group and because the total protein was not controlled, statistics were not carried out.

Table 5.3 PrP values (including C1) detected by DELFIA® detected for ovine PrP transgenic mice

Transgenic line	(PrP ng/ml)
KVRQ 1	0.3, 0.4
KVRQ 2	0.5, 0.5
KVRQ 3	0.1, 0.3
KARQ 1	0.5, 0.6
KARQ 2	0.6, 0.8
Tg338 1	5.0, 5.3
Tg338 2	7.2, 7.3

PrP^C quantification is expressed in ng/ml. Full-length PrP^C was measured using the DEFLIA® and this was added to the mean amount of C1 which was measured from the Western blot results to get an estimate total PrP^C value **(Full-length PrP^C + (Full-length PrP^C x % C1)** = Total PrP^C. Each sample was assayed in duplicate and the corresponding means calculated. Each sample was repeated twice n = 2. KVRQ group contained 3 mice (n = 3), ARQ group contained two mice (n = 2) and the Tg338 group contained two mice (n = 2).

5.2.5 Detection of C1 in PrP glycosylation deficient mice

To investigate whether glycosylation of PrP is important for alpha cleavage, PrP-glycosylation deficient mice were analysed. The presence of the mutant and wild type alleles were confirmed by PCR (2.2.5). Visual inspection of Western blots suggests that the electrophoretic mobility of mono-glycosylated PrP differs between G1 and G2 mice (Figure 5.9 B and C) this has been shown previously (Cancellotti *et al*., 2005).

Without deglycosylation PrP truncated fragments were observed in the G1, G2 and G3 mice when 6H4 was used for detection (Figure 5.7A, lanes 2, 3, 4 and Figure 5.7B, lanes 3, 4, 7, 8). When BC6 (epitope 147-155, Figure 3.11) was used for detection PrP truncated fragments were also observed in the 129/Ola controls as well as the G1, G2 and G3 mice (Figure 5.7 C, lanes 5 and 9). The PrP truncated fragment observed in the G3 mice is predicted to be the C1 fragment. The PrP truncated fragments observed in the G1, G2 and 129/Ola mice are predicted to be C1 with one or two carbohydrates attached. The PrP truncated band in the G1 mice (Figure 5.7A, lane 1 and Figure 5.7 C, lane 2 and 6) is positioned at a slightly higher molecular weight compared to the G2 mice this would correspond to the mono-glycosylated form of PrP.

When brain homogenates from the PrP-glycosylation deficient mice are fully deglycosylated with PNGase the complete content of C1 fragment is revealed in the G1, G2 and G3 mice (Figure 5.8A, lanes 5, 6, 7 and Figure 5.8B, lane 2, 8). However, when 6H4 is used for detection after deglycosylation sometimes C1 is present (Figure 5.8B, lane 3) and other times C1 is not present (Figure 5.8A, lanes 11-13).

In conclusion the C1 fragment is processed in all three PrP glycosylation deficient mice. This suggests that the carbohydrates are not required for alpha cleavage.
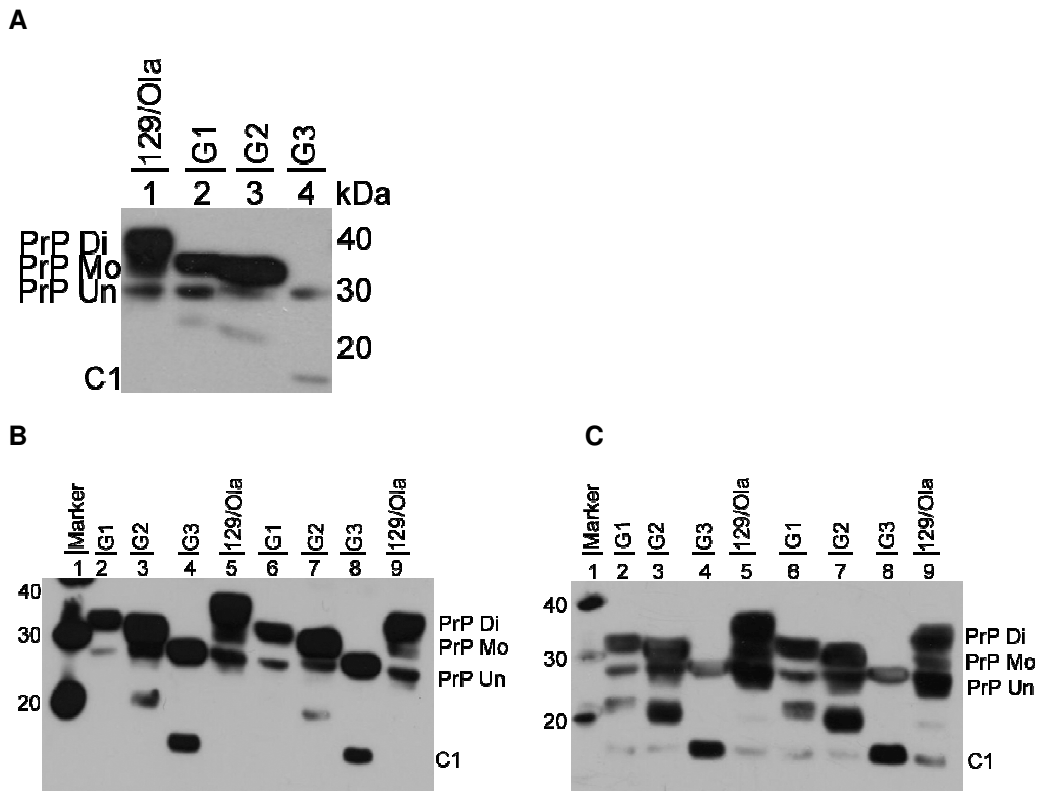
Figure 5.7 Western blot profiles of PrP glycosylation deficient mice

(A) This blot demonstrates the difference in the electrophoretic mobility of monoglycosylated PrP^C from G1 and G2 transgenic mice. In the G1 and G2 mice there is an extra lower molecular weight band present at ~ 35 kDa and 34 kDa. In the G3 mice there is also an extra lower molecular weight band that is equivalent in size to the C1 fragment at ~ 18 kDa. PrP^C was detected using monoclonal antibody 6H4. (B) This blot highlights the same aspects as blot A however there is no extra molecular weight band in the G1 mice. Only the C1 band is observed in the G3 mice. PrP^C was detected using monoclonal antibody 6H4. (C) Membrane in (B) was stripped and re-probed using monoclonal antibody BC6. This blot shows that there are lower molecular weight bands seen in the G1, G2 and 129/ola control mice at ~ 20 and 18 kDa as well as the G3 mice. 5 μl of 10 % brain homogenate was loaded for each sample. Each experiment was repeated twice (n = 2).
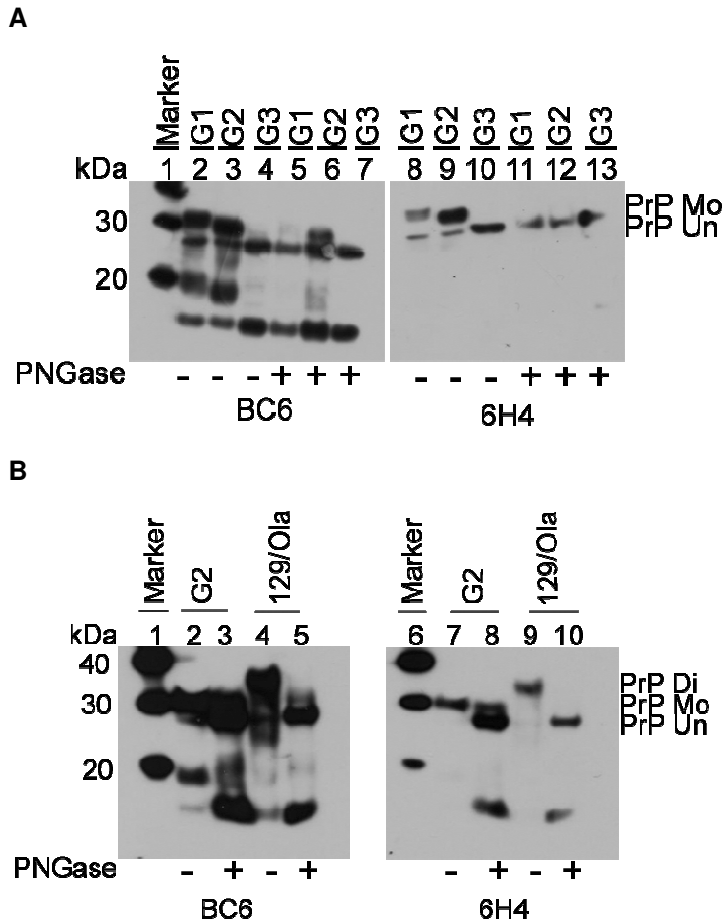
Figure 5.8 Western blot profiles of PNGase deglycosylated total brain proteins from PrP glycosylation deficient mice detected using monoclonal antibodies BC6 and 6H4

(A) The C1 fragment is present in the PNGase positive and negative samples (lane 2-7) when BC6 is used for detection. An extra band is observed in the PNGase negative samples for G1 and G2 mice (lane 2, 3). The C1 fragment is not present in the PNGase positive samples (lane 11, 12, 13) when 6H4 is used for detection. (B) The C1 fragment is present in the PNGase positive and negative samples (lane 2, 3, 4, 5) for G2 and 129/Ola mice when BC6 is used for detection. The C1 fragment is present in the PNGase positive samples (lane 8, 10) for G2 and 129/Ola mice when 6H4 is used for detection. 5 µl of 10% brain homogenate was loaded for each lane (A and B). Monoglycosylated PrP^C detected at 24-25 kDa, unglycosylated PrP^C at 26 kDa, C1 band at ~ 18 kDa and another lower molecular weight band at ~ 20 kDa. Each experiment was repeated twice (n = 2).

5.3 Discussion

Our results from the various transgenic mouse lines show that alpha cleavage occurs in all PrP transgenic mice. The primary sequence of PrP does not appear to significantly affect the processing of this cleavage. There are 25 amino acid differences within the coding region of PrP between sheep and mice. Only one of these changes is close to the alpha cleavage site, this change is found at codon 112 where methionine in sheep is replaced for leucine in mice at the equivalent position (codon 108). As there is little difference between the primary PrP sequence between mice and sheep within the alpha cleavage region it may not be surprising that alpha cleavage occurred in these transgenic mice. One can therefore conclude that all these mouse lines are useful models for alpha cleavage. However, the KB6 mice which have six octapeptide repeats had more C2 compared to the KB5 mice which have only five octapeptide repeats. As beta cleavage occurs within the octapeptide region, the amount of octapeptides may have an effect on beta cleavage.

Interestingly a recent paper by Oliveira-Martins *et al*., (2010) suggests that the amino acid sequence near the cleavage site of PrP is not critical for alpha cleavage. This group showed that alpha cleavage occurred in a variety of cell lines which was only disrupted when large areas of the central domain were deleted. These results show that the central domain of PrP is important but the protease which may be responsible for this cleavage is not overly dependent on sequence specificity. It may be that other domains of PrP may be important for alpha cleavage.

A study by Haigh *et al*., (2009) show that the primary sequence of PrP around the alpha cleavage site is important for proteolytic processing as cells expressing mouse PrP produced significantly more C1 compared to cells expressing human PrP. Studies on Alzheimer's disease report that the distance from the membrane may be more important for protease activity rather than the amino acid sequence of the protein (Sisodia *et al*., 1990, Maruyama *et al.*, 1991 Novak, 2004). The experiments presented here indicate that there is no reduction between cleavage of mouse and sheep PrP sequence.Therefore sequence appears to be less relevant for alpha cleavage than other as yet unknown factors.

Transgenic mice that over-expressed PrP showed an increase in the percentage of C1 compared to wild type mice and mice that expressed close to normal levels of PrP. Interestingly over-expressing mice have shorter incubation periods compared to wild type mice, however it is currently impossible to separate the possible effect of C1 levels from the well established dosage effect of full-length prion protein. In a recent paper Huang *et al.*, (2007) showed that over-expression of PrP in Tg mice lead to a myopathy that correlates with high levels of C1. These results suggest that an increase in C1 may be detrimental and may also have an influence on scrapie disease susceptibility. However, Tg338 mice are healthy up to 600 days of age (Hunter *et al.*, personal communication) and no abnormalities in muscle tissue have been reported.

KVRQ mice are not the best model of VRQ sheep when considering alpha cleavage. In relative terms sheep with the VRQ/VRQ genotype produce more C1 as a percentage of total PrP^C than the KVRQ transgenic mouse line, however only 4 animals were used per group. KARQ and Tg338 mice provide a much better representation of alpha cleavage when compared to ARQ/ARQ and VRQ/VRQ sheep. However, as Tg338 mice over-express PrP they are compromised models for that reason.

Results from the DELFIA® analysis showed that Tg338 mice express 10 times more PrP compared to KVRQ and KARQ mice, however this result was less than the 25x difference that was quantified via Western blotting (Figure 5.3). As Western blot analysis is only semi-quantitative it is likely that this result was an overestimate and that the DELFIA® result provided a more accurate measurement. Interestingly Tg338 mice have been previously quoted to have 8-10 fold more PrP compared to a normal sheep, however the amount of PrP detected in the Tg338 in this study was more equivalent to the levels detected in a sheep (4.2.7) although direct comparisons cannot be made as the total amount of protein loaded in the DELFIA® assay was not controlled. However, as the levels of PrP^C detected in the KARQ and KVRQ were relatively equal it can be assumed that a higher degree of accuracy was achieved with the DELFIA® compared with the Western blot quantification.

The data presented here also show that all three PrP-glycosylation deficient mice produce the C1 fragment. Antibody 6H4 did not consistently detect C1 in the PrP-glycosylation deficient mice. The percentage of C1 produced in PrP-glycosylation deficient mice was more than 50% of the total PrP^C signal Figure 5.8 (A and B) when BC6 was used for detection. When BC6 was used for the detection of C1, 129/Ola mice had equivalent levels of C1 as the PrP-glycosylation deficient mice (Figure 5.8 B).

PrP Truncated fragments were observed in G1, G2 and 129/Ola mice when BC6 was used for detection Figures 5.7 C lanes 1, 3, 5, 6, 7, 9, Figure 5.8 A lanes 2, 3, 6 and Figure 5.8 B lanes 2, 3, 4, 5. Truncated forms were also observed in G1 and G2 mice with 6H4 (Figures 5.7 A lane 2, 3, Figure 5.7 B lane 3, 7). The truncated fragments in the glycosylation deficient mice are likely to reflect glycosylated forms of C1 and possibly C2 as these fragments were not observed in the G3 mice.

In conclusion, the carbohydrates are not required for PrP alpha cleavage as G3 mice which have the glycosylation sites abolished were able to cleave C1 efficiently. It is known that the trafficking of PrP^C is altered in G3 mice as PrP^C is mainly localised internally within the Golgi (Cancellotti *et al.*, 2005) but it appears that the alpha cleavage is independent of reaching the plasma membrane or recycling. Although subtle effects on alpha cleavage cannot be excluded from these experiments

Chapter 6 Detection of the alpha cleavage fragment C1 in ovine and murine peripheral tissues using novel monoclonal antibodies

6.1 Introduction

In previous chapters PrP^C and the C1 fragment have been successfully detected from ovine and murine brain samples using a simple lysis extraction (2.1.1). However, this method was not successful for the detection of PrP^C from peripheral tissues even after a purification step was added to the extraction method (2.1.2). Within the literature PrP^C has been detected from many murine peripheral tissues but the number of studies that have detected PrP^C from ovine peripheral tissues are limited. In the few studies that have been able to detect PrP^C from ovine peripheral tissue, immunoprecipitation methods have been used to concentrate levels of PrP^C (Horiuchi *et al.*, 1995, Moudjou *et al.*, 2001) before detection by Western blotting.

As well as detecting PrP by Western blotting Moudjou *et al.*, (2001) also quantified the amount of PrP from peripheral tissues using solid phase EIA methodology. This group found consistently high levels of PrP expression in the lung, heart and skeletal muscle in three different ovine PrP genotypes. These quantification results were supported by their Western blot data that showed high levels of PrP detection for these tissues, although much less compared to the brain control (Moudjou *et al.*, 2001). Interestingly ARQ/VRQ animals contained the highest PrP expression levels for five out of the nine peripheral tissues tested. However, a link between genotype and quantification levels of PrP could not be investigated fully as only a small number of animals were used in this study.

Unfortunately neither Horiuchi *et al.*, (1995) or Moudjou *et al.*, (2001) examined the presence of C1 in these tissues. In order to expand upon C1 data that had already been collected for ovine brain tissue various peripheral tissues were also tested for the presence of the C1 fragment. It is of interest to investigate the percentage of C1 in peripheral tissues to see if alpha cleavage is processed in the same way in different tissue types and to see if the percentage of alpha cleavage varies accordingly.

In an attempt to concentrate PrP from peripheral tissues immunoprecipitation was carried out using three different IgM (IgM κ) monoclonal antibodies. Antibodies were kindly supplied by (M. Jones, Blood Transfusion Service Scotland). Antibodies that were chosen for immunoprecipitation of PrP^C were (IgM) H1.8, H3.2 and H3.3. The epitopes of these antibodies are directed against the C-terminal end of PrP (Figure 3.11, Figure 6.1). The epitopes of these antibodies were the main reason that these antibodies were chosen, so that the amount of full-length PrP and C1 in peripheral tissues could be compared. Antibody 6H4 which had previously been used for Western blotting was not used for immunoprecipitation as other groups had shown that 6H4 gave poor results for the immunoprecipitation of PrP^C (unpublished data). A novel IgG antibody (BC6, Kind gift from Sandra McCutcheon, Roslin Institute) which had also been used previously for Western blotting was also used for immunoprecipitation of PrP^C.

Antibody H3.2 was of particular interest as this antibody was able to immunoprecipitate human PrP^C, PrP^{Sc} and PrP^{Res} (Jones *et al*., 2009) from human brain tissue. Because this antibody had been able to precipitate PrP^C from human tissue it was hoped that it would be able to immunoprecipitate PrP^C from ovine tissue. H1.8 and H3.3 were chosen as these antibodies were able to detect mouse PrP^C and PrP^{Sc} by Western blotting (Jones *et al*., 2009). For previous results of these antibodies for immunoprecipitation and Western blotting see Table 6.1 (A, B and C).

The IgM antibodies were produced by Michael Jones and his group at the Blood Transfusion Service, Scotland. The IgM antibodies were raised against human platelets (Jones *et al*., 2005). By raising these antibodies against human platelets it was hoped that they may be useful in the development of a blood-screening assay for PrP. PrP null mice were used to overcome the problem of immune tolerance (Andrievskaia *et al*., 2006, Jones *et al*., 2009). However, the animal spleens (B-lymphocytes) were fused together with a myleoma cell that did express PrP, although at very low levels (Kim *et al*., 2003, Jones *et al*., 2009). For each fusion the mouse chosen received a final intravenous boost of 50 mg pHuPrP in phosphate-buffered saline (PBS) and was sacrificed 4 days later (Jones *et al*., 2009). The effect of this

PrP expression is unknown but may have some effect on antibody production and stability of the cell lines.

Hybridoma supernatants were routinely screened for the production of antibody binding to pHuPrP by ELISA. Hybridoma cell lines producing anti-pHuPrP antibodies were single cell cloned, expanded, and cryopreserved according to standard procedures (Jones *et al.*, 2009).

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Ovine and murine tissue was available for the characterisation of these antibodies. These antibodies provide the potential of visualising PrP^C and C1 on a Western blot from uninfected peripheral tissues and also provide data to compare against infectious samples. Scrapie samples were also tested to see if these antibodies could precipitate PrP^{Sc}.

Table 6.1 A

MAb	PrP^C	PrP^{Sc}
H1.8	Weak	Weak
H3.2	Yes	Yes
H3.3	No	Yes

Table 6.1 B

MAb	RecHuPrP	NPL -PK	NAPS -PK	ADB -PK	vCJDB -PK	vCJDB +PK
H1.8	No	No	No	No	No	No
H3.2	Weak	No	No	No	No	No
H3.3	No	No	No	No	No	No

Table 6.1 C

MAb	RecMoPrP	Normal brain- PK	Scrapie brain + PK
H1.8	Yes	Yes	Yes
H3.2	Yes	Yes	Yes
H3.3	Yes	Yes	Yes

Table 6.1 (A) **Immunoprecipitation of human PrP^C and PrP^{Sc}**. Data from (Jones *et al*., 2009). Immunoprecipitation data for MAb H1.8, H3.2 and H3.3 which were tested on Alzheimer's control brain (PrP^C), and vCJD brain with PK digestion (PrP^{Sc}). (B) **Detection of human PrP^C and PrP^{Sc} by Western blotting.** Data from V. McLoughlin, M.Phil thesis (2009). Western blot data for MAb H1.8, H3.2 and H3.3 which were tested on normal platelet lysate (NPL), normal activated platelet supernatant (NAPS), Variant Creutzfeldt Jacob disease (vCJD) and Alzheimer's disease brain (ADB). (C) **Detection of mouse PrP^C/PrP^{Sc} by Western blotting.** Data from V. McLoughlin, M.Phil thesis (2009). Western blot data for MAb H1.8, H3.2 and H3.3, which were tested on recombinant mouse PrP (RecMoPrP), normal mouse brain and scrapie infected mouse brain.

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H1.8            147–166

Human   DRYYRENMHRYPNQVYYRPM
Ovine   --------Y----------V
Ovine   ----H---Y----------V
Murine  --------Y----------V


H3.2            157–176

Human   YPNQVYYRPMDEYSNQNNFV
Ovine   ---------V-Q--------
Ovine   ---------V-R--------
Murine  ---------V-Q--------


H3.3      186–205 (Glycosylation site at 196)

Human   HTVTTTTKGENFTETDVKMM
Ovine   ----------------I-I-
Murine  --------------------
```

Figure 6.1 PrP epitopes for MAbs H1.8, H3.2 and H3.3

Bold letters indicate amino acid change. Glycosylation site present at amino acid 196. This
work was undertaken by Victoria McLoughlin (Data was taken from her M.Phil).

6.2 Results

6.2.1 Single cell cloning to stabilise cell lines

In the first attempt to revive the monoclonal cell lines H3.3 and H3.2 problems were encountered as they were dying shortly after they were removed from LN_2 storage. Whereas H3.3 cells grew quickly after another fresh batch was taken out of LN_2 storage, H3.2 cells took approximately 3 months to grow To support cell growth mixed thymocyte media (MTM) and R20 was added to the cells when they were in $25cm^2$ flasks and in 96 well plates. MTM media provides the cells with extra growth factors (Micklem *et al*., 1987). These cell lines were previously frozen down without a cell viability count and never re-grown, so the amount of viable cells may have been initially low, however it is likely that the cell lines are unstable.

Single cell cloning of H1.8, H3.2 and H3.3 from flasks at 5 cells/well and 1 cell/well was carried out to clone more stable cell lines. Colony counts and screening of cloning plates by ELISA was also carried out. Figure 6.2 shows an ELISA result for cell line H1.8 for the production of IgM antibody. Cells from the 1 cell/well cloning plates were grown up to $75cm^2$ flasks, and then they were frozen down for future characterisation studies. One vial from each cell line and each frozen batch was taken back out of LN_2 shortly after storage and was re-grown to check that the cell line was stable.
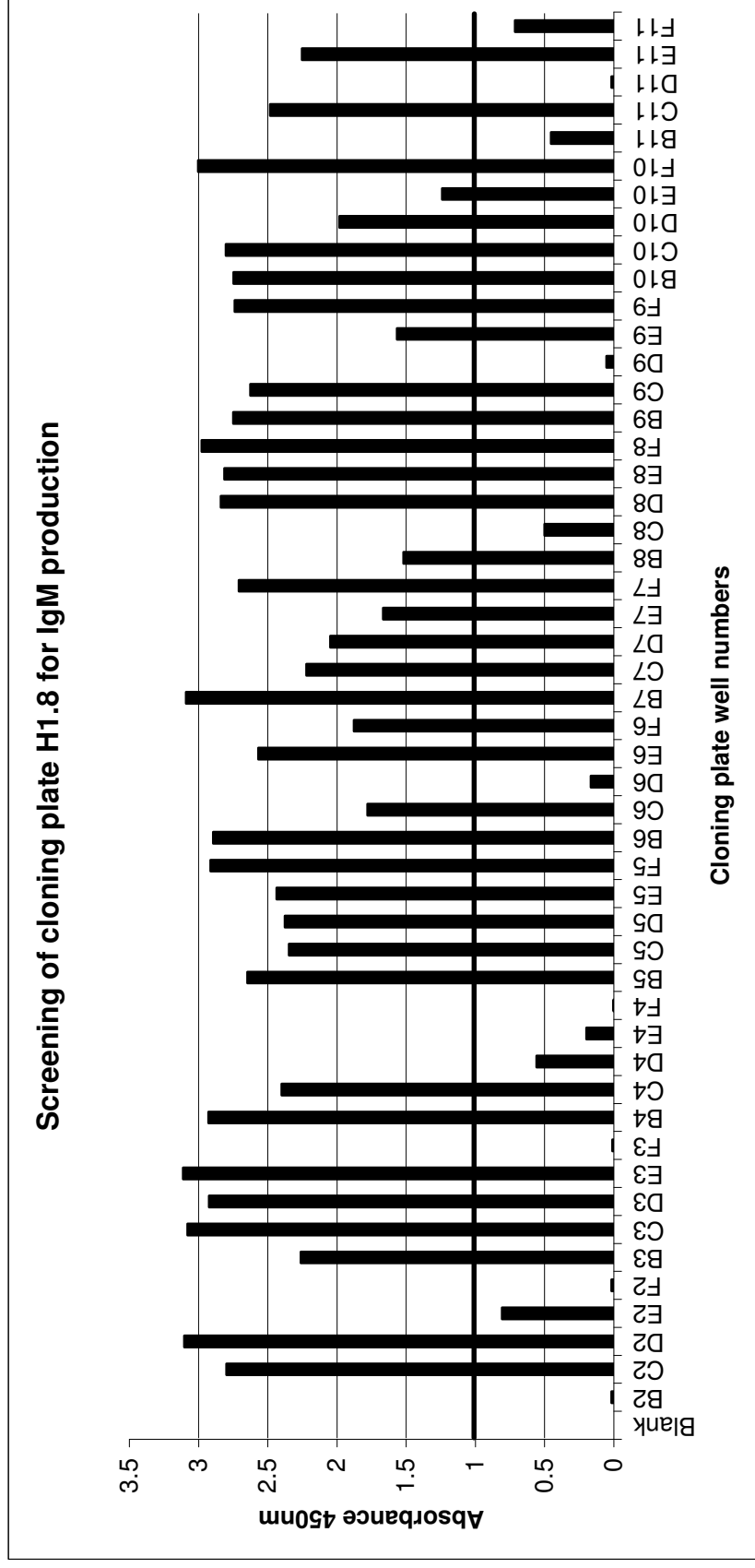
Figure 6.2 Histogram showing the binding absorbance at 450nm (y-axis) of IgM antibodies present in the cloning plate of H1.8 cell line

Results were determined by ELISA with the microwells coated with anti-mouse IgM. A goat anti-mouse (μ-chain specific) IgM HRP conjugate was used as a secondary antibody and the assay was stopped 10 minutes after the addition of the substrate solution. Any results that were 1 or above were taken to be positive for IgM.

6.2.2 Quantification of purified IgM isotype monoclonal antibodies by ELISA

Purification of all three IgM antibodies was carried out using ammonium sulphate and quantification of the final IgM concentration was undertaken using a simple ELISA. Two fold serial dilutions of mouse IgM standard (Sigma) [200ng/ml to 1.56ng/ml] were prepared along with serial dilutions of the MAb [1:1000 to 1:256000]. The mean absorbance (450nm) for each standard was plotted against the corresponding IgM concentration to produce a standard curve from which the IgM concentration of the test sample was determined. The total IgM concentration for each antibody can be seen in Table 6.2.

Table 6.2 Total IgM concentration for cell line H1.8, H3.2 and H3.3

MAb	Concentration (mg/ml) of MAb
H1.8	4060
H3.2	3440
H3.3	2740

Summary of all MAbs grown up *in vitro* from LN_2 frozen stocks. The names of the IgM MAbs are described in the first column, followed by the antibody concentration of the MAb (mg/ml).

6.2.3 Immunoprecipitation of PrP from vCJD and normal human brain homogenate using IgM MAbs H1.8, H3.2 and H3.3

The three IgM MAbs and a non-PrP related IgM isotype control were evaluated for their ability to immunoprecipitate PrP^C from uninfected human brain and PrP^{Sc} from vCJD infected brain (Figure 6.3). Human samples were used a control to confirm previous published results and to ensure that the antibodies were working correctly. MAb H3.2 immunoprecipitated from both brain homogenates, suggesting that this MAb recognises both normal and prion disease associated forms of HuPrP (Figure 6.3, lanes 1 and 2). MAb H3.3 only immunoprecipitated from non-PK digested vCJD brain, suggesting that this MAb displays selectivity towards abnormal disease associated PrP (Figure 6.3, lane 4). Neither MAb H1.8 nor the IgM control appeared to immunoprecipate any forms of PrP (Data not shown); these results support published work by Jones *et al*., (2009).

A lower truncated fragment at ~ 16 kDa was visible in both uninfected and infected samples (Figure 6.3, lanes 1, 2 and 4). This lower molecular fragment was not observed or mentioned in the previous publication (Jones *et al*., 2009). Even though protease inhibitors are used in the preparations of brain homogenates it could be possible that this truncated fragment is degradation of PrP^C caused by *post-mortem* lysis of the brain tissue. The storage and handling conditions of this particular brain sample was unknown. It is also possible that this fragment may also be a novel truncated fragement of PrP.
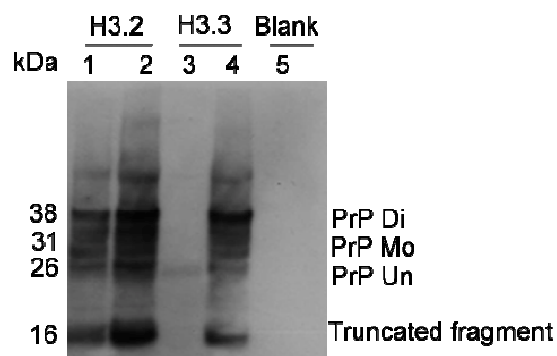
Figure 6.3 Western blotting of PrP^C immunoprecipitated from uninfected human brain homogenate and PrP^{Sc} from vCJD infected human brain homogenate

Uninfected (lanes 1 and 3) and vCJD infected (lanes 2 and 4), human brain homogenates (10%) without proteinase K (PK) treatment were incubated with anti-PrP antibodies H3.2 and H3.3 coupled to magnetic beads Immunoprecipitated PrP^C was detected by antibody 3F4. 20 µl of immunoprecipitate was loaded for each sample. The diglycosylated form of PrP was present at ~ 36 kDa, monoglycosylated PrP at ~ 31 kDa and unglycosylated PrP at ~ 26 kDa. An unidentified truncated fragment migrates at ~ 16 kDa. This experiment was repeated twice (n = 2).

6.2.4 Immunoprecipitation of PrP from scrapie infected ovine brain and uninfected ovine brain

The three IgM MAbs and a non-PrP related IgM isotype control were also evaluated for their ability to immunoprecipitate PrP^C from uninfected ovine brain and PrP^{Sc} from scrapie infected ovine brain. When antibody 6H4 was used for detection MAb H3.2 immunoprecipitated PrP^C and PrP^{Sc}, however only di and unglycosylated forms of PrP were detectable (Figure 6.4 lane 1 and 2). MAb H3.3 immunoprecipitated only unglycosylated PrP (Figure 6.4 lane 3). This result suggests that H3.2 recognised both normal and prion disease associated forms of Ovine PrP and that H3.3 only recognises normal Ovine PrP. Neither MAb H1.8 nor the IgM control immunoprecipated any forms of PrP (Data not shown).

These results were repeated but this time antibody BC6 was used for detection. This IgG antibody shares the same epitope as 6H4. Although 6H4 and BC6 have the same PrP sequence, they may have different conformations for PrP. In the first experiment H1.8, H3.2 and H3.3 immunoprecipitated PrP^C from ovine ARQ/ARQ brain homogenate (Figure 6.5A, lanes 4, 6 and 8). Only H3.3 immunoprecipitated PrP^{Sc} from scrapie infected ovine ARQ/ARQ brain homogenate (Figure 6.5A, lane 9). The IgM control did not immunoprecipitate any form of PrP. The genotype of the homogenate used in the 6H4 experiment (Figure 6.4) was unknown so; these results may be due to genotypic differences. This experiment was repeated, this time all three MAbs immunoprecipitated PrP^C (Figure 6.5B, lanes 3-5). PrP^{Sc} was not immunoprecipitated by any of the antibodies (Figure 6.5B, lanes 7-9).

To follow up the idea that there may be some genotypic differences uninfected ARR/ARR brain homogenate was incubated with H1.8, H3.2 and H3.3 and PrP detected by Western blot using BC6 (Figure 6.6). All three MAbs immunoprecipitated PrP^C (Figure 6.6, lanes 3, 5 and 7). As a negative control dynabeads were incubated in the presence of brain homogenate but in the absence of antibody, this was negative as expected (Figure 6.6 lane 9).

Figure 6.4 Western blot of PrP^C immunoprecipitated from uninfected ovine brain homogenate and PrP^{Sc} immunoprecipitated from scrapie infected ovine brain homogenate

Uninfected (Lanes 1 and 3) and scrapie infected (Lanes 2 and 4) ovine brain homogenates (10%) without proteinase K (PK) treatment were incubated with anti-PrP antibodies (H3.2 and H3.3) coupled to magnetic beads. Blank lanes indicate sample buffer only. Immunoprecipitated PrP was detected by antibody 6H4. 20 µl of immunoprecipitate was loaded for each sample. The diglycosylated form of PrP present at ~ 36 kDa, monoglycosylated PrP at ~ 31 kDa, unglycosylated PrP ~ at 26 kDa. This experiment was repeated twice (n = 2).

Figure 6.5 Western blot of PrP^C immunoprecipitated from uninfected ovine brain homogenate and PrP^{Sc} immunoprecipitated from scrapie infected ovine brain homogenate

(A) Uninfected and scrapie infected brain homogenates (10%) from ARQ/ARQ homozygotes. Uninfected (lanes 2, 4, 6 and 8) and scrapie infected (lanes 3, 5, 7 and 9). (B) Uninfected and scrapie infected brain homogenates (10%) from ARQ/ARQ homozygotes. Uninfected (lanes 3, 4 and 5) and infected (lanes 7, 8 and 9). Ovine brain homogenates without proteinase K (PK) treatment were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Magnetic beads (Dynabeads) were incubated as a negative control in the presence of brain homogenate. Blank lanes indicate sample buffer only. Immunoprecipitated PrP was detected using monoclonal antibody BC6. 20 μl of immunoprecipitate was loaded for each sample. The diglycosylated form of PrP was present at ~ 36 kDa, monoglycosylated PrP at ~ 31 kDa and unglycosylated PrP at ~ 26 kDa. Both these experiments were carried out once (n = 1).
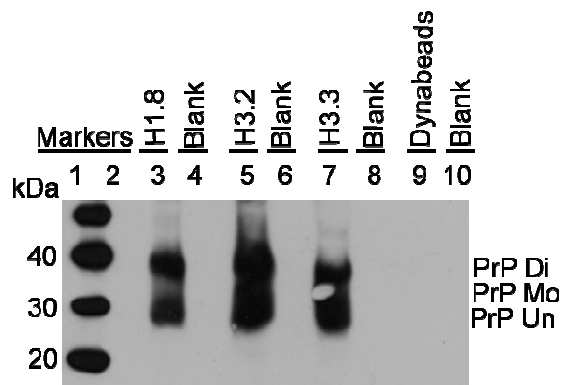
Figure 6.6 Western blot of PrP^C immunoprecipitated from uninfected ovine brain homogenate from an ARR/ARR homozygote

Uninfected (Lanes 3, 5 and 7) ovine brain homogenates (10%) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Blank lanes indicate sample buffer only. Magnetic beads (Dynabeads) were incubated as a negative control in the presence of brain homogenate but in the absence of anti-PrP antibody. Immunoprecipitated PrP was detected by antibody BC6. 20 µl of immunoprecipitate was loaded for each sample. The diglycosylated form of PrP^C was present at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa, unglycosylated PrP^C at ~ 26 kDa. This experiment was carried out once (n = 1).

6.2.5 Immunoprecipation of PrP from uninfected ovine mesenteric lymph node

As PrP^C was successfully immunoprecipitated from the brain of uninfected sheep the next experiment investigated whether PrP^C could be immunoprecipitated from the mesenteric lymph node (MLN). Mesenteric lymph node was chosen as this peripheral tissue is infected during scrapie infection and was available for the same animals used in the brain analysis. Messenger RNA expression within lymph node has been calculated to be equivalent to levels within certain areas of the brain (Moudjou *et al.*, 2001), therefore it may be possible to imunoprecipitate PrP^C from this tissue.

After incubation and precipitation with anti-PrP antibodies (H1.8, H3.2 and H3.3) PrP was not detected in the MLN by Western blotting (Figure 6.7, lanes 4, 6 and 8). A brain sample was used as a positive control and as expected PrP was detected after immunoprecipitation with all three MAbs (Figure 6.7, lanes 5, 7 and 9). As a negative control MLN and brain homogenates were incubated in the presence of a non PrP related IgM antibody. Unexpectedly the IgM control immunoprecipitated PrP^C from the brain sample (Figure 6.7, lane 3); this suggests that non-PrP related antibodies may be capable of immunoprecipitating PrP, this has been observed previously for PrP^{Sc} (Morel *et al.*, 2004) or that occasionally PrP^C when present at high concentrations can adhere to the beads or other material and can be carried over through the washes. The IgM control was negative for the MLN sample (Figure 6.7, lane 2).

To investigate whether PrP was not detected in the MLN due to low levels of starting material a repeat of this experiment was carried out by increasing the starting concentration of the material of the MLN from 1mg/ml to 10mg/ml. After an increase in concentration PrP detection in the MLN was still negative (Data not shown).

Figure 6.7 Western blot of PrP^C immunoprecipitated from uninfected ovine brain homogenate and PrP^C immunoprecipitated from uninfected ovine MLN

Uninfected brain (B) and MLN (M) was from an ARR/ARR homozygote. Brain (Lanes 3, 5, 7 and 9) and MLN (Lanes 2, 4, 6 and 8) homogenates (10%) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads or a corresponding IgM isotype (non-prion related). Immunoprecipitated PrP was detected by antibody BC6. 20 µl of immunoprecipitate was loaded for each sample (Brain and MLN). The diglycosylated form of PrP^C was present at ~ 36 kDa, monoglycosylated PrP^C at ~ 31 kDa and unglycosylated PrP^C at ~ 26 kDa for brain samples only. No PrP^C was detected in the MLN samples.

As IgM antibodies H1.8, H3.2 and H3.3 were unable to immunoprecipitate PrP^C from the mesenteric lymph node IgG antibody BC6 was used for immunoprecipitation of PrP^C ovine MLN and ovine brain as a control. Unfortunately, after immunoprecipitation of normal sheep brain, scrapie sheep brain and ovine mesenteric lymph node there was a lot of unspecific staining (Figure 6.8 A). No unspecific staining was observed when dynabeads were incubated in the presence of normal brain (Figure 6.8 A, lane 10) and normal MLN (Figure 6.8 A, lane 11) but in the absence of BC6.

Antibody BC6 was also incubated as a control in the presence of lysis buffer only and unspecific staining was observed in this sample (Figure 6.8 A, lane 9). Therefore the unspecific binding must be related to either the capture (BC6) or the detector antibody (P4). To determine whether the unspecific staining was due to the capture or detector antibody the membrane was stripped of the detector antibody and the membrane re-probed with the secondary antibody only (Figure 6.8 B). The Western blot image in Figure 6.8 B shows that after stripping of the detector antibody (P4) most of the unspecific staining remains. This suggests that the capture antibody BC6 is responsible for the unspecific staining.

In an attempt to eliminate the unspecific staining and to see if PrP could be detected, BC6 which is used at a concentration of 1mg/ml for immunoprecipitation was diluted (1:10 and 1:20) before it was used for immunoprecipitation. The Western blot image in Figure 6.8 C shows that unspecific binding is reduced compared to Figure 6.8 A and B but BC6 is unable to immunoprecipitate PrP^C from ovine brain and MLN and PrP^{Sc} from scrapie infected ovine brain. In conclusion, IgG antibody BC6 was unable to immunoprecipitate any forms of PrP.
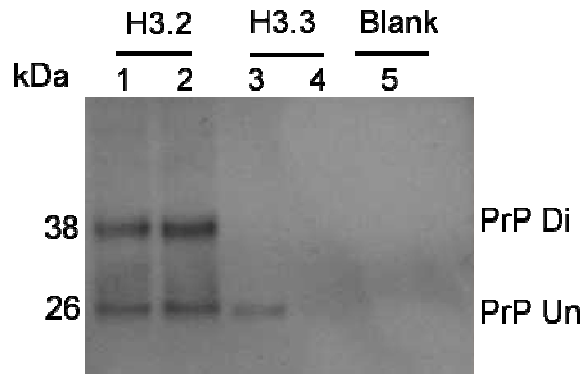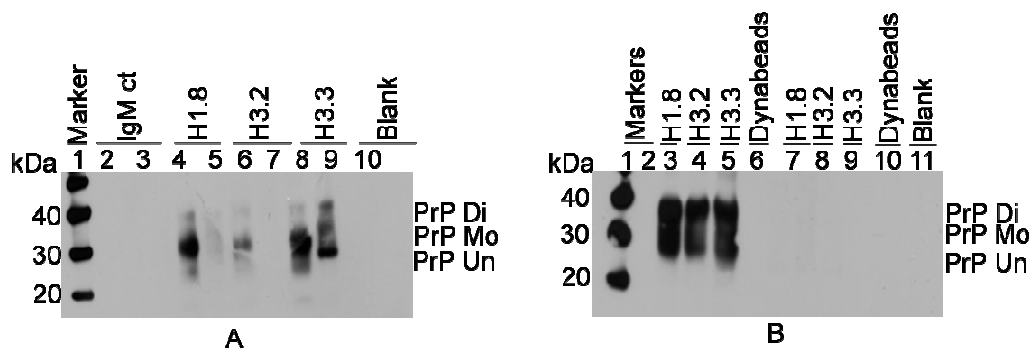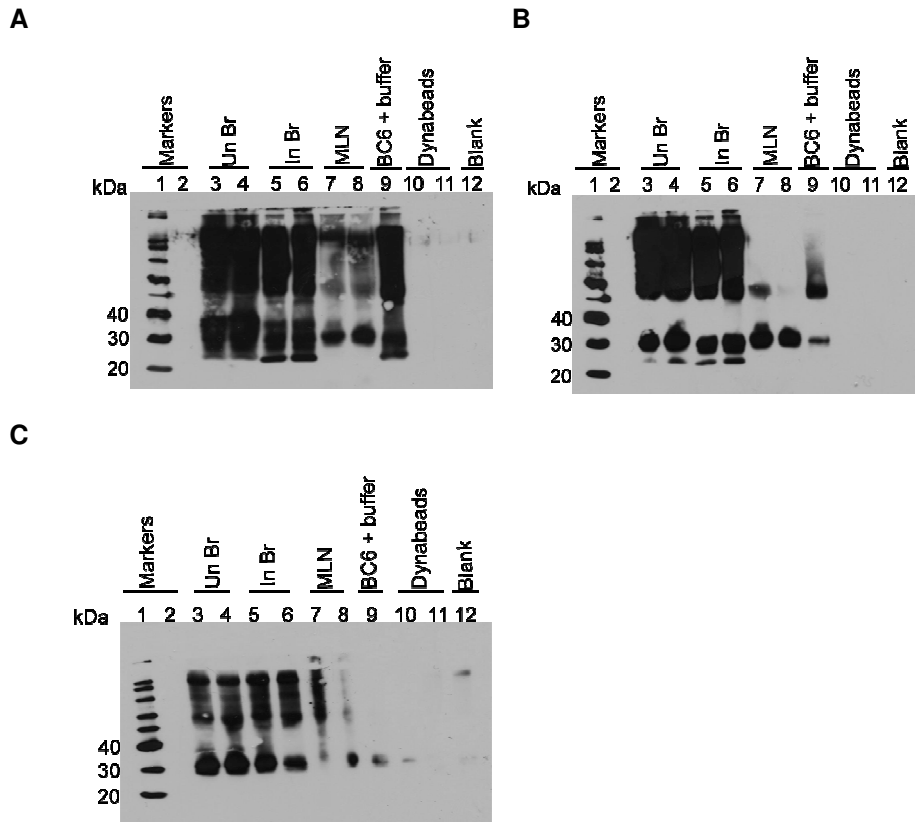
Figure 6.8 Western blots of PrP^C immunoprecipitated from uninfected ovine brain homogenate, MLN homogenate and PrP^{Sc} immunoprecipitated from scrapie infected brain homogenate

(A) Uninfected brain (Un Br) (lanes 3 and 4), scrapie infected brain (In Br) (lanes 5 and 6) and MLN (lanes 7 and 8) were incubated with anti-PrP antibody BC6 coupled to magnetic beads (Dynabeads). Magnetic beads (Dynabeads) were incubated as a negative control in the presence of brain homogenate (lane 10) and in the presence of MLN (lane 11) but in the absence of anti-PrP antibody. Protein was detected using monoclonal antibody P4. (B) The Western blot in (A) was stripped using restore stripping reagent and re-probed with secondary antibody only. (C) Repeat of blot (A) but a 1:10 (lanes 3, 5, 7, 9 and 11) and 1:20 (lanes 4, 6, 8 and 10) dilution of BC6 was carried out before immunoprecipitation. 10 µl of immunoprecipitate was loaded for each sample. This experiment was carried out once (n = 1).

154

6.2.6 Immunoprecipation of deglycosylated PrP and C1 from uninfected mesenteric lymph node

Deglycosylation experiments on PrP^C immunoprecipitated from ovine ARQ/ARQ mesenteric lymph node (MLN) were carried out in the hope of detecting the C1 fragment from this peripheral tissue. Brain tissue was also deglycosylated as a positive control. Treatment of PrP^C with PNGase resulted in simplifying the PrP^C profile into one or two bands. The top band corresponds to unglycosylated PrP^C and the bottom band migrates at the same size as the C1 fragment. Figure 6.9 (lanes 5, 7 and 9) shows that H1.8, H3.2 and H3.3 are able to immunoprecipitate PrP^C and the C1 fragment from brain tissue after deglycosylation, although the bands for the C1 fragment are very faint. A band at the same size as the unglycosylated form of PrP is present in the MLN after immunoprecipitation with H1.8, H3.2 and H3.3 (Figure 6.9, lanes 6, 8 and 10) but the C1 fragment is not detectable. There is also a band in the IgM control for both brain and MLN homogenates (Figure 6.9, lanes 2 and 3). This band is slightly higher than the band for unglycosylated PrP therefore it is likely that this band is not PrP.

The experiment was repeated with homogenates from an ARR/ARR homozygote animal. Figure 6.10 (lanes 5, 7 and 9) shows that H1.8, H3.2 and H3.2 are able to immunoprecipitate PrP^C and the C1 fragment from the brain after deglycosylation. Antibody H1.8 immunoprecipitated the most C1 although complete deglycosylation has not occurred and an extra band is seen above the C1 band (indicated by * on blot). A doublet is also seen for immunoprecipitation of PrP^C from the brain with antibody H3.2 and H3.3 (Figure 6.10, lanes 9 and 7). All three MAbs were unable to precipitate PrP^C and C1 from the MLN (Figure 6.10, lanes 6, 8 and 10). There are two bands in the IgM control for brain and one band in the IgM control for MLN (Figure 6.10, lanes 2 and 3).

In conclusion, it appears that H1.8, H3.2 and H3.3 can immunoprecipitate PrP^C and the C1 fragment from the brain of uninfected sheep although the intensity of PrP signal can vary between animals and between the different antibodies. These three antibodies do not precipitate unglycosylated PrP^C or the C1 fragment from the MLN.

A
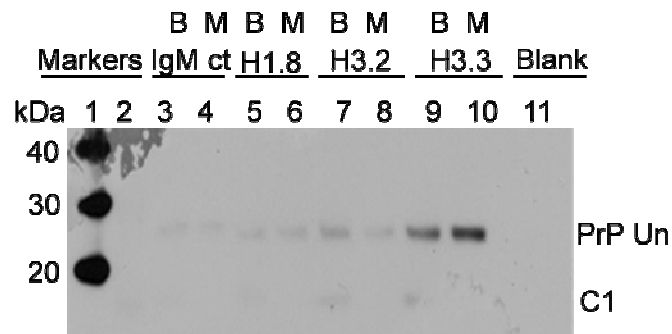
B
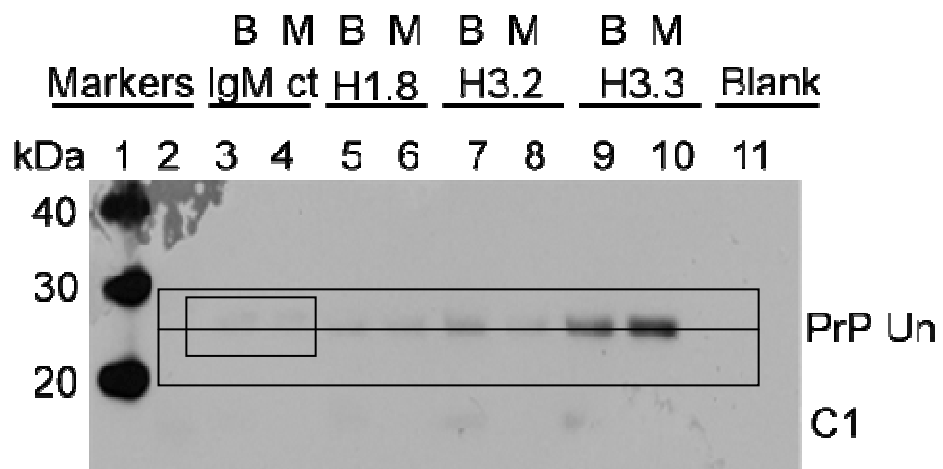
Figure 6.9 Sensitivity of PrP^C from the brain and MLN to treatment with PNGase F

A) Normal sheep brain homogenate (B) (10%) (lane 3, 5, 7 and 9) and MLN (M) homogenate (10%) (4, 6, 8, and 10) from ARQ/ARQ homozygote was treated with PNGase F and were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) or to a corresponding IgM isotype (Non-PrP related) coupled to magnetic beads. Blank lane indicates sample buffer only. Immunoprecipitated PrP^C and C1 were detected by Western blot with monoclonal antibody BC6. B) Enlargement of image in A. This image has been highlighed by a box to emphasise the size difference between bands in lane 3 and 4 compared to other bands in the image. The unglycosylated PrP band (lanes 5-10) migrates at ~ 25 kDa while a band of ~ 26 kDa is observed in lanes 3 and 4. The C1 band migrates at ~ 18 kDa. 20µl of immunoprecipitate was loaded for each sample (Brain and MLN). This experiment was carried out once (n = 1).

A
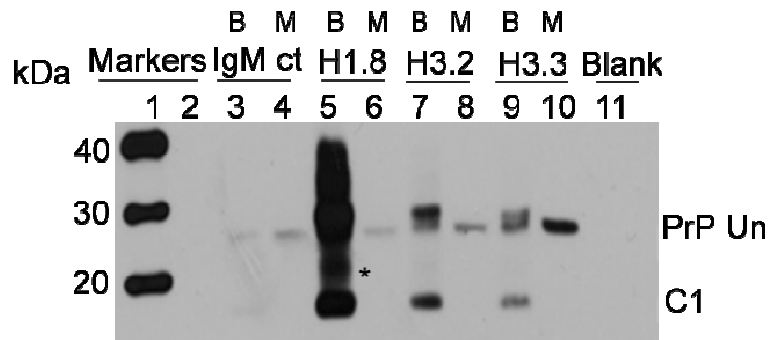
B
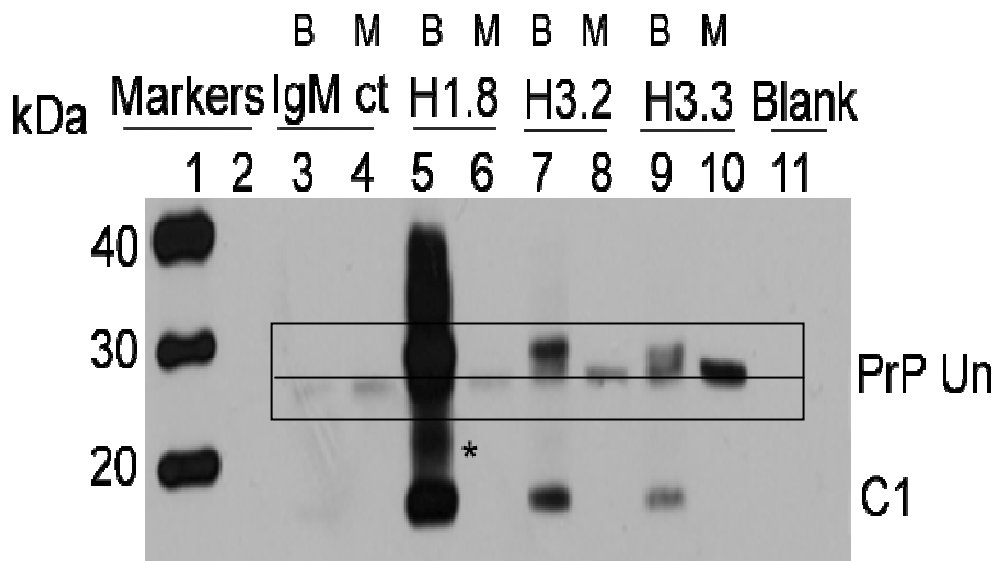
Figure 6.10 Sensitivity of PrP^C from the brain and MLN to treatment with PNGase F

A) Normal sheep brain homogenate (10%) (lane 3, 5, 7 and 9) and MLN homogenate (10%) (lane 4, 6, 8 and 10) from ARR/ARR homozygote was treated with PNGase F and were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) or to a corresponding IgM isotype (Non-PrP related) coupled to magnetic beads. Blank lane indicates sample buffer only. Immunoprecipitated PrP^C and C1 were detected by Western blot with BC6. A band above the C1 fragment (most likely to be the C2 fragment) is indicated by * on the blot B) Enlargement of image in A. This image has been highlighted by a box to emphasise the doublet band observed in lanes 5, 7 and 9. Unglycosylated PrP^C migrates at ~ 25 kDa the doublet band observed in lane 5, 7 and 9 migrates at ~ 27 kDa, Band indicated by * migrates at ~ 20 kDa while the C1 band migrates at ~ 18 kDa. 20µl of immunoprecipitate was loaded for each sample (Brain and MLN). This experiment was carried out once (n = 1).

6.2.7 Immunoprecipitation of PrP from uninfected murine tissues and ME7 infected brain

As MAbs H1.8, H3.2 and H3.3 were unable to immunoprecipitate PrP^C from ovine peripheral tissues we decided to investigate whether PrP^C could be detected from the periphery of Tg338 mice. These antibodies were first tested for their ability to immunoprecipitate murine PrP^C. All three IgM MAbs successfully immunoprecipitated PrP^C from 129/Ola mouse brain homogenate (Figure 6.11 lane 1, 2 and 3) after detection with antibody BC6. As PrP^C was successfully detected from a 129/Ola brain homogenate it was thought that Tg338 (over-expressing ovine Tg mice) could be used as a model for detecting ovine PrP^C within peripheral tissues. In the next set of experiments Tg338 brain and liver were incubated with MAbs H1.8, H3.2 and H3.3 and 129/Ola brain used as a positive control. Proteins were detected by Western blotting using BC6.

PrP^C was successfully detected from Tg338 brain and 129/Ola brain homogenates as expected (Figure 6.12, lanes 3-8) after immunoprecipitation with H1.8, H3.2 and H3.3. PrP^C was detected from Tg20 brain after immunoprecipitation with H1.8 only (Figure 6.15, lane 9). A band was detected in the Tg338 liver lanes for all three antibodies (Figure 6.12, lanes 9-11; Figure 6.13, lanes 9-11,) at approximately 25 kDa however as this band migrated slightly lower than unglycosylated PrP^C it would suggest that this band is IgM light chain and not PrP. An attempt was also made to immunoprecipitate PrP^C from Tg338 spleen homogenate however this was unsuccessful and only unspecific staining was detected (Figure 6.14, lanes 1-3 and Figure 6.15, lanes 3-5).

To investigate whether MAbs H1.8, H3.2 and H3.3 can immunoprecipitate PrP^{Sc} from murine brain tissue these antibodies were incubated with ME7 infected C57/B mouse homogenate. After three immunoprecipitation attempts none of the IgM antibodies was able to immunoprecipitate PrP^{Sc} from scrapie infected mouse brain homogenate (Figure 6.13, lanes 3-5; Figure 6.14, lanes 4-6 and Figure 6.15, lanes 6-8). Table 6.3-Table 6.6 provide a summary of the immunoprecipitation results for human, ovine, murine tissues.

In conclusion, MAbs H1.8, H3.2 and H3.3 only immunoprecipitated from uninfected murine brain homogenate, suggesting that these MAbs display selectivity towards the normal isoform of PrP. H1.8, H3.2 and H3.3 were unable to immunoprecipitate PrP^C from over-expressing transgenic spleen and liver which suggests that the expression level of these tissues were too small or the confirmation of PrP^C in these tissues does not allow the antibodies to bind to the protein.
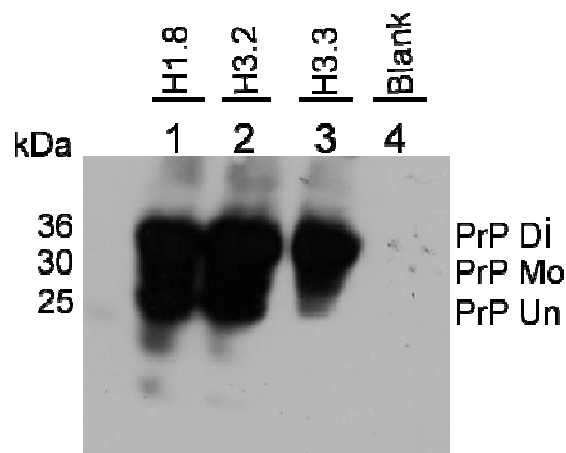
Figure 6.11 Immunoprecipitation of PrP^C from 129/Ola brain homogenate (10%)

Uninfected 129/Ola brain homogenate (10%) was incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads and analysed by Western blot. Blank lanes indicate sample buffer only. Immunoprecipitated PrP^C was detected by antibody BC6. The diglycosylated form of PrP^C was at ~ 36 kDa, monoglycosylated PrP^C at ~ 30 kDa and unglycosylated PrP^C at ~ 25 kDa. 20µl of immunoprecipitate was loaded for each sample.
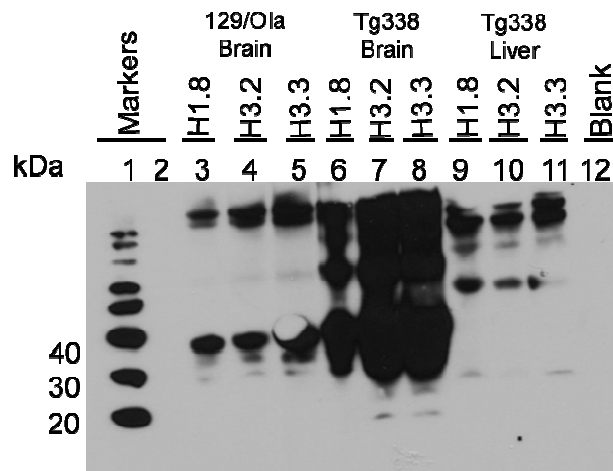
Figure 6.12 Western blot of PrP^C from brain and liver immunoprecipitates

Uninfected murine brain homogenate (10%) (Lanes 3-5), Tg338 brain homogenate (10%) (lanes 6-8) and Tg338 liver homogenate (10%) (lanes 9-11) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Blank lane indicates sample buffer only. Immunoprecipitated PrP was detected by antibody BC6. The diglycosylated form of PrP^C was at ~ 36 kDa, monoglycosylated PrP^C at ~ 30 kDa and unglycosylated PrP^C at ~ 25 kDa. 20µl of immunoprecipitate was loaded for each sample (brain and liver). This experiment was carried out twice (n = 2).
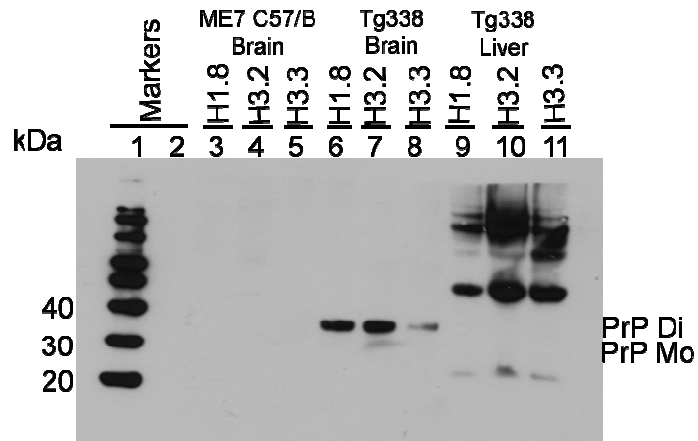
**Figure 6.13 Western blot of PrP$^{\text{C}}$ and PrP$^{\text{Sc}}$ brain and liver immunoprecipitates**

ME7 infected C57/B brain homogenate (10%) (lanes 3-5) uninfected Tg338 brain homogenate (10%) (lanes 6-8) and uninfected Tg338 liver homogenate (10%) (lanes 9-11) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Immunoprecipitated PrP$^{\text{C}}$ was detected by antibody BC6. The diglycosylated form of PrP$^{\text{C}}$ migrated at ~ 36 kDa. No PrP$^{\text{Sc}}$ was detected in the ME7 infected brain homogenate (lanes 3-5) and no PrP$^{\text{C}}$ detected in the Tg338 liver homogenate (lanes 9-11). 20µl of immunoprecipitate was loaded for each sample (brain and liver). This experiment was carried out twice (n = 2).
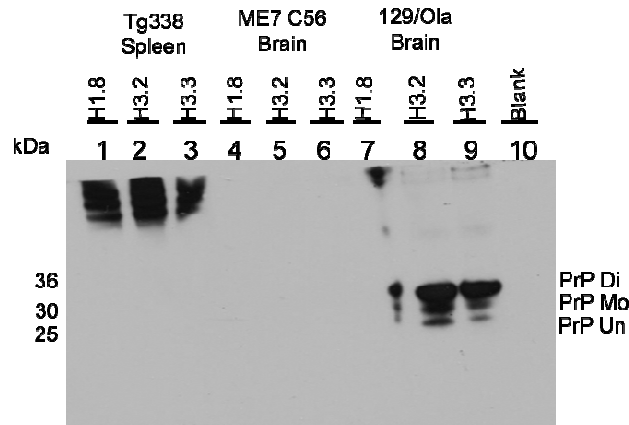
Figure 6.14 Western blot of PrP^C and PrP^{Sc} brain and spleen immunoprecipitates

Uninfected Tg338 spleen homogenate (10%) (lanes 1-3), ME7 infected C57/B mouse brain homogenate (10%) (lanes 4-6) and uninfected 129/Ola mouse brain homogenate (10%) (lanes 7-9) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Immunoprecipitated PrP^C was detected by antibody BC6. Blank lane indicates sample buffer only. The diglycosylated form of PrP^C migrated at ~ 36 kDa, monoglycosylated PrP^C at ~ 30 kDa and unglycosylated PrP^C at ~ 25 kDa. No PrP^C was detected in the spleen of Tg338 mouse (lane 1-3), and no PrP^{Sc} was detected in the ME7 infected brain homogenate (lanes 4-6). 20 µl of immunoprecipitate was loaded for each sample (brain and spleen).This experiment was carried out twice (n = 2)
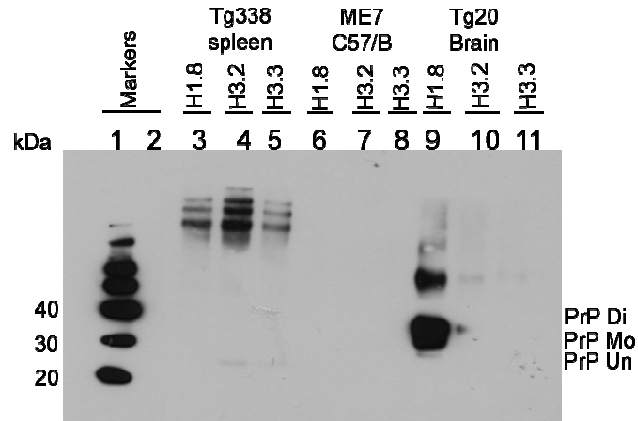
Figure 6.15 Western blot of PrP^C and PrP^{Sc} spleen and brain immunoprecipitates

Uninfected Tg338 spleen homogenate (10%) (lanes 3-5), ME7 C57/B mouse brain homogenate (10%) (lanes 6-8) and uninfected Tg20 brain homogenate (10%) (lanes 9-11) were incubated with anti-PrP antibodies (H1.8, H3.2 and H3.3) coupled to magnetic beads. Immunoprecipitated PrP^C was detected by antibody BC6. The diglycosylated form of PrP^C migrated at ~ 36 kDa, monoglycosylated PrP^C at ~ 30 kDa and unglycosylated PrP^C at ~ 25 kDa. No PrP^C was detected in the spleen of Tg338 mouse (lane 1-3), and no PrP^{Sc} was detected in the ME7 infected brain homogenate (lanes 4-6). 20µl of immunoprecipitate was loaded for each sample (brain and spleen). This experiment was carried out once (n = 1).

Table 6.3 Immunoprecipitation of ovine PrP^C and PrP^{Sc}

MAb used for immunoprecipitation	Brain	MLN	Brain
	PrP^C	PrP^C	PrP^{Sc}
H1.8	Yes	No	No
H3.2	Yes	No	Yes
H3.3	Yes	No	Yes

Immunoprecipitation data for MAb H1.8, H3.2 and H3.3 which were tested on uninfected ovine brain (PrP^C Brain), uninfected mesenteric lymph node (PrP^C MLN) and scrapie infected brain without PK digestion (PrP^{Sc} brain).

Table 6.4 Immunoprecipitation of wild type murine PrP^C and murine PrP^{Sc}

MAb used for immunoprecipitation	Brain	Brain
	PrP^C	PrP^{Sc}
H1.8	Yes	No
H3.2	Yes	No
H3.3	Yes	No

Immunoprecipitation data for MAb H1.8, H3.2 and H3.3 which were tested on uninfected murine brain (PrP^C 129/Ola brain) and ME7 scrapie infected brain without PK digestion (PrP^{Sc}).

Table 6.5 Immunoprecipitation of murine PrP^C from Tg338 and Tg20 mouse models

MAb used for Immunoprecipitation	PrP^C Tg338 Brain (8x)	PrP^C Tg338 Liver (8x)	PrP^C Tg338 Spleen (8x)	PrP^C Tg20 Brain (10x)
H1.8	Yes	No	No	Yes
H3.2	Yes	No	No	No
H3.3	Yes	No	No	No

Immunoprecipitation data for MAb H1.8, H3.2 and H3.3 which were tested on uninfected Tg338 brain, Tg338 liver, Tg338 spleen and Tg20 brain.

Table 6.6 Immunoprecipitation of human PrP^C and PrP^{Sc}

MAb used for Immunoprecipitation	Uninfected brain	vCJD brain
	PrP^C	PrP^{Sc}
H1.8	No	No
H3.2	Yes	Yes
H3.3	Yes	Yes

Immunoprecipitation data for MAb H1.8, H3.2 and H3.3 which were tested on uninfected human brain homogenate (PrP^C) and vCJD infected brain homogenate without PK digestion (PrP^{Sc}).

6.3 Discussion

The aim of this part of the study was to concentrate PrP^C from peripheral tissues using immunoprecipitation techniques. IgM antibodies H1.8, H3.2 and H3.3 were chosen for immunoprecipitation of PrP from ovine and murine tissues. IgM antibodies are normally difficult to use in immunoprecipitation due to their pentameric structure and also because they do not bind well to protein A or protein G. To overcome these problems IgM antibodies were purified using ammonium sulphate and anti-IgM dynabeads (Invitrogen) were used in the immunoprecipitation reactions.

As a control ovine brain homogenate was used to observe whether these antibodies could immunoprecipitate PrP^C as these antibodies had never been tested for immunoprecipitation on ovine tissue previously. The brain was used as PrP expression in the brain is much greater than in peripheral tissues (Han *et al*., 2006, Gossner *et al*., 2009). PrP^C was successfully immunoprecipitated from ovine brain tissue using H1.8, H3.2 and H3.3 from as little as 1 mg starting material. Unfortunately PrP^C could not be precipitated from ovine mesenteric lymph node by any of the IgM MAbs. The reason for the MLN being negative for PrP may be due to low protein expression within the tissue or it may be that PrP conformation within the MLN may not allow these antibodies to bind to the native protein. It is likely that the starting material of the MLN was at too low (10 mg) a concentration as Moudjou *et al*., (2001) used 50mg of tissue to detect PrP in various peripheral tissues and Horiuchi *et al*., (1995) required 200mg of tissue to detect PrP in lymph node. Increasing the loading concentration of the MLN was not investigated further due to time constraints and limited tissue availability.

In one case two bands were observed in ovine brain homogenates after deglycosylation and one band observed in the MLN homogenate (Figure 6.10). It can be speculated that the two bands observed in the brain samples correspond to unglycosylated PrP and C1, and the one band observed in the MLN is unspecific protein. Another possibility is that the band observed in all MLN is IgM light chain although IgM heavy chain was not present. It is also possible that the top band

observed in the doublet for brain samples is unglycosylated PrP and unglycosylated PrP in the MLN runs at a slightly lower molecular weight. Although this control was not carried out in this experiment, dynabeads incubated in the presence of the IgM antibodies only, does not produce any reaction which suggests that the band observed in the MLN is not IgM light chain and is unspecific protein. If there had been more time it would have been possible to use an anti-light chain antibody to be confirm these results. Therefore in conclusion it is unknown whether C1 is present in the MLN or as more experiments would need to be carried out to confirm this and due to time restraints this was not possible. It is likely that C1 is produced in many peripheral tissues as ADAMs that are thought to be responsible for PrP alpha cleavage are widely expressed within the periphery (Novak, 2004). It is however possible that PrP and ADAM proteases may not interact at the cellular level within certain peripheral tissues.

As a way of trying to precipitate PrP^C from ovine peripheral tissues, over-expressing ovine, transgenic mice (Tg338) were used as a model. Unfortunately, PrP^C was not detected from Tg338 spleen or liver but was detected from Tg338 brain. Not being able to immunoprecipitate PrP^C from the liver was expected as other groups have shown that the PrP^C MRNA in the liver is expressed at very low levels (Horiuchi *et al*., 1995, Moudjou *et al*., 2001). These results indicate that PrP^C may not be expressed in the liver as PrP^C could not be detected in our over-expressing transgenic mouse model.

MAbs H1.8, H3.2 and H3.3 only immunoprecipitated PrP from uninfected murine brain homogenate, suggesting that these MAbs displays selectivity towards the normal form of PrP. H1.8, H3.2 and H3.3 were unable to immunoprecipitate PrP^{Sc} from ME7 infected mouse brain, although this experiment was only carried out once (n = 1).

If there had been more time it would have been beneficial to test these IgM antibodies for the detection of PrP^C by Western blotting and used an IgG antibody for immunoprecipitation. IgG antibody BC6 was tested for immunoprecipitation of

PrP^C, PrP^{Sc} from ovine brain and mesenteric lymph nodes with unsuccessful results when P4 was used for detection by Western blotting.

In conclusion, the detection of PrP fragmentss within peripheral tissues may provide further understanding about the biochemistry and function of PrP. Unfortunately, full-length PrP^C and the C1 fragment was not detected in the peripheral tissues of ovine sheep and ovine transgenic mouse models. Initial characterization of these three IgM antibodies suggests that there are some interesting properties associated with these antibodies that can be further investigated in future characterisation studies.

Chapter 7 Final discussion

7.1 Alpha cleavage and TSE disease

The main aim of this study was to investigate the role of alpha cleavage in scrapie disease susceptibilbility with the idea that total full-length PrP^C, total C1 and the ratio between full-length PrP^C and C1 may be linked to differences in scrapie susceptibility.

A significant difference in the amount of C1 was observed between some of the genotypes after REML analysis, although the amount of C1 did not correlate with incubation period with SSBP/1. There was also no significant difference between the percentage of C1 between the highly susceptible VRQ/VRQ and resistant ARR/ARR animals; this suggests that the amount of alpha cleavage in these animals does not play a major role in defining disease susceptibility. The data presented here is supported by *in vitro* data from (Sabuncu *et al.*, 2005) who show that VRQ and ARR variants do not display significant differences in the amount of C1 production. Their conclusion that resistance to infection was most likely not due to differences in alpha cleavage but may be associated with differences in solubility between the two variants may be applicable to sheep.

The percentage of C1 varied depending on the brain region in sheep (Chapter 4). The percentage of C1 was higher in the cortex and thalamus than the cerebellum and medulla. If ADAM proteases are responsible for alpha cleavage it may be that these proteases are expressed at different levels within different brain areas, a clue that could become important in the search for an enzyme or enzymes responsible for alpha cleavage. The cerebellum and medulla consistently produced lower percentages of C1 across five different genotypes making genotype much less of a factor that modulates alpha cleavage in different brain areas. In terms of scrapie pathogenesis the frontal cortex is the last area to accumulate PrP after injection with SSBP/1 scrapie and this area shows the most C1. This result would fit in with the model that more C1 is protective. On the other hand, the cerebellum shows almost no pathology but has very little C1.

As only roughly defined areas of the brain were taken for this analysis future studies should consider to collect more precise sections of the brain. This may require different collection methods for sheep brain tissue. It may then be possible to correlate areas of high or low C1 with strain targeting. Currently within the TSE genetics group full-length PrP^C and C1 levels are being quantified in seven areas of the sheep brain (Lauren Campbell, personal communications).

Neural cells taken from the cerebellum of Tg338 transgenic mice and maintained in cell culture produced a high percentage of C1 (Cronier *et al*., 2004). As Tg338 mice are highly over-expressing PrP mice it is possible that as a consequence more C1 is produced in all or some brain areas. This has already been shown to be the case in the frontal cortex of Tg338 mice *in vivo* (Chapter 5). The dominance of C1 over full-length PrP^C produced in these cells is contradictory to our *in vivo* data that shows dominance for full-length PrP^C. However as these two systems are very different it is difficult to compare results. Variation in protein detection via Western blotting can be caused by the use of different antibodies (Kuczius *et al*., 2007a,b) but it is equally possible that differences between various studies arise from the handling and storage of samples.

The possibility that other codons within the open reading frame of PrP may influence alpha cleavage and disease susceptibility can not be ruled out. All of the animals used in the genotype study were sequenced between codons 86 and 202. As the animals were mostly derived from closed flocks for which fairly complete data exist about all polymoprhisms it is very unlikely that any of the animals used had additional sequence variation in the unsequenced PrP regions. No additional polymorphisms were found in these animals with the exception of codons 112 and 141. The frequency of these two polymorphism – keeping the 136 position constant- was too low to study disease association with alpha cleavage.

Naturally occurring polymorphisms do exist around the alpha cleavage site in sheep but are not likely to have a major effect on C1 production as Oliveira-Martins *et al*.,

(2010) suggest based on their *in vitro* models. Data by Oliveira-Martins *et al*., (2010) also showed that point mutations within the hydrophobic domain have some effect on alpha cleavage, but do not completely abolish the production of the C1 fragment. It appears that the size of the central domain is more important than the amino acid sequence for PrP alpha cleavage; this has been shown by Wegner *et al*., (2002) and Oliveira-Martins *et al*., (2010).

It is probable that other regions of PrP may be important for alpha cleavage and not just the sequence directly around the alpha cleavage site. It may be that the proteases responsible for alpha cleavage are dependent on the distance from the cell membrane rather than sequence specificity (Novak, 2004). Sequences further C-terminal of the alpha cleavage site would be potential candidates but they have not yet been studied for their effects on C1 processing and function. It has been hypothesised that PrP catalyses its own release from full-length PrP and can function as an α-secretase (Abdulla, 2001). This theory proposes that two histidines at codon 96 and 111 participate in a metalloprotease assembly which is then able to initiate cleavage. There is no evidence from the experimental data in this thesis to support the hypothesis that PrP is able to cleave itself. Crucially, data from Oliveira-Martins *et al*., (2010) show that alpha cleavage still occurs after histidines at codons 96 and 111 are changed to glutamic acid or deleted which argues against the self cleavage model.

The hydrophobic domain of PrP has been subject to debate as to whether or not this area is involved in conversion. Transgenic mice with particular deletions within the hydrophobic region of PrP (Baumann *et al*., 2007, Li *et al*., 2007, Shmerling *et al*., 1998) develop a neurodegenerative condition different from TSE disease. This condition can be reversed with the re-introduction of wild type PrP. This suggests that the central domain of PrP may provide some protection against neurodegeneration. However the transmembrane region 1 (TM1) which is located within the central domain of PrP can predispose the protein to take up alternative pathogenic topologies (Hegde *et al*., 1998a, Stewart & Harris, 2005).

The sheep samples studied here contained animals of various ages and the association of age with alpha cleavage was investigated. The percentage of C1 detected in new born lambs (aged 0-5 days) was much less than in young lambs aged 100-320 days and in adult sheep. The percentage of C1 detected in the 100-320 days old lambs was ~30%, which is equivalent to the percentage found in adult sheep. This suggests that there may be a very early developmental effect as the percentage of C1 increased between a few days of age and a few hundred days of age. It appears that PrP is expressed first followed by expression of the alpha cleavage enzyme. This suggests that the enzyme that is responsible for alpha cleavage is independent from PrP, countering the argument for self-cleavage. Knowledge of this delay in the alpha cleavage activity could help to characterise the enzyme.

In terms of disease there appears to be an association with age and susceptibility to disease, for example most cases of variant Creutzfeld Jacob disease occur in young adults (Ghani et al., 2003) most cattle that developed BSE were infected as young calves (Arnold & Wilesmith, 2004) and it has been suggested that young lambs are more susceptible than adults to natural scrapie infection (Diaz et al., 2005). However, this rule does not apply to every species as neonatal mice are more resistant to scrapie infection than adult mice (Ierna et al., 2006) although this has been attributed to lack of spleen development in the neonatal mice. As milk has been shown to be infectious (Konold et al., 2008) and as there is a low percentage of C1 in new born lambs then the likelihood of natural infection may be higher as there is more available full-length PrP. However, this effect does not last long as the percentage of C1 increases dramatically in weaned animals.

To follow on from the data collected on uninfected animals it might be of interest to evaluate levels of cleavage in TSE infected models to see if there is any correlation between the amount of alpha cleavage and PrP^{Sc} accumulation. In conclusion the association between the percentage of C1, susceptible genotypes and ultimately susceptibility to scrapie is not strong and may only play a minor part in disease

7.2 Normal function of alpha cleavage

As well as investigating the role of the C1 fragment in terms of disease susceptibility this thesis has also been interested in the mechanisms and function behind this proteolytic event. The cellular location of PrP alpha cleavage has previously been shown to occur in a late compartment of the Golgi as there was a reduction in the amount of C1 after blocking the exit of PrP from the endoplasmic reticulum (Walmsley *et al*., 2009). Here, PrP glycosylation mice were used as a model to gain further understanding about the cellular location of PrP cleavage. G3 mice were of particular interest as a model because PrP is located intracellularly within the Golgi apparatus of these mice (Cancellotti *et al*., 2005). PrP in the G1 and G2 mice is located on the cell surface (Cancellotti *et al*., 2005) but cellular trafficking may also be impaired in these mice. C1 was detected in all three PrP-glycosylation deficient mice which suggest that cleavage must occur within the Golgi compartment. The ratios of full-length PrP to C1 in these G-mice were very similar to the 129/Ola wildtype mice which indicate that N-glycosylation is neither essential nor detrimental for alpha cleavage. It also suggests that unglycosylated PrP follows similar pathways as glycosylated PrP through the Golgi compartment.

Throughout this thesis the effects of the C1 fragment have been investigated. Over-expression of the C1 fragment displays pro apoptotic function (Sunyach *et al*., 2007). The C1 fragment has also been linked to MAPK signalling pathways (Haigh *et al*., 2009). The corresponding N1 fragment may also be of importance as it has been shown to have neuroprotective properties as it protects cells against staurosporine induced apoptosis and down regulates p53 transcription (Sunyach *et al*., 2007). Unfortunately we were unable to detect N1 via Western blotting; it may be that during preparation of brain material N1 is more prone to degradation. However, one can be certain that the amount of N1 produced is equal to the amount of C1 produced as these fragments are produced from the same cleavage event.

The metalloproteases ADAM 9 (Cisse *et al*., 2005), 10 (Endres *et al*., 2009), 17 (TACE) (Cisse *et al*., 2005, 2007, 2008) and more recently ADAM 23 (Costa *et al*., 2009) have been suggested to mediate production of the C1 and N1 fragments within

the Golgi apparatus (Shyng *et al*., 1995). ADAM 17 and ADAM 10 have also been shown to be involved in cleavage of the APP (Alzheimer's precursor protein) (Buxbaum *et al*., 1998). However, despite some evidence of ADAM involvement in cleavage of the prion protein the direct mechanisms of these proteases are still undefined and should form a basis for future work in sheep PrP biology.

There are other similarities between the cleavage of PrP^C and APP, for example both cleavage of PrP^C and APP occurs within a toxic domain of the protein. C-terminal fragments of PrP^C that include the 106-126 sequence can cause neurotoxicity in cultured neurones (Ettaiche *et al*., 2000, Haik *et al*., 2000). Alpha cleavage of the prion protein disrupts this region which may provide some protection against toxicity. Cleavage of APP within the Aβ region of APP prevents the formation of the Aβ toxic peptide. Both these cleavage events are thought to occur by alpha secretases, for example ADAM 10; although this is not known for certain for PrP^C.

The APP protein can also be cleaved by Beta-secretase 1 enzyme (BACE-1), which allows the release of the toxic Aβ peptide. In 2007 Parkin and co-workers reported that overexpression of PrP^C in human neuroblastoma cells lead to a decrease in Aβ, whilst PrP^C silencing lead to an increase in Aβ production (Parkin *et al*., 2007, Kellet *et al*., 2009). It was noted further that the effect of PrP^C on Aβ production was due to inhibition of BACE-1 and therefore a decrease in the amount APP cleavage. This group found that the N-terminal region of PrP^C and localisation on lipid rafts are required for PrP^C to have an inhibitory effect on BACE-1 (Kellet et al., 2009). It has been suggested that PrP^C may interact via glycosaminoglycans (such as heparin) with binding sites on BACE1, which may restrict the access of BACE-1 to APP (Araki 2010, Kellet *et al*., 2009). This group concluded that PrP^C is protective against Alzheimer's disease as it reduces the formation of Aβ peptides. In terms of therapeutics the aim has been to diminish beta-amyloid production by secretases inhibition although this has not been as successful as it has been hoped. As alpha cleavage of PrP^C reduces the amount of full-length PrP^C it may be possible that this cleavage event could block some of the interactions observed between PrP^C and APP.

Cleavage of the prion protein can also occur when the cell is subject to oxidative stress (McMahon *et al.*, 2001, Watt *et al.*, 2005). This cleavage is termed β cleavage and occurs within the octapeptide repeat region which allows the hydrophobic region of PrP^C to remain intact. Unlike the N1 and C1 fragments the C2 and N2 fragments of β cleavage are not associated with pro or anti apoptotic function (Sunyach *et al.*, 2007). It can be speculated that these fragments may be produced in response to disease as there is evidence that a fragment of similar size to C2 is protease resistant (Owen *et al.*, 2007) and may therefore be involved in prion conversion. Although C2 can not be created out of C1 it is possible that C1 could be produced by C2. This suggests that some interaction may occur between these two fragments which may be related to disease susceptibility.

In PrP overexpressing mice the C2 fragment was observed. The KB6 mice that over-express PrP^C with six octapeptide repeats had consistently more C2 on average than all other PrP^C overexpressing mice (Table 5.2). The KB6 mice also had more C2 than normal expressing KB5 mice with five octapeptide repeats (data not shown). It may be that the increase in C2 in the KB6 mice is due to the over-expression of PrP, or it may be due to the amount of octapeptide repeats. As beta cleavage occurs within the octapeptide repeats it may be worth investigating the effect that these repeats have on cleavage.

7.3 Alpha cleavage in transgenic mice

Alpha cleavage of PrP has been shown to occur in a variety of species and this study has proven that it is active in transgenic mice expressing murine, ovine and bovine PrP at various levels. Transgenic mice that over-express PrP show an increase in the percentage of C1 when compared to wild type mice. These mice can live until old age without any adverse phenotypic effects therefore it is unlikely that an increase in C1 is detrimental to these mice (Hunter, unpublished observations). However, (Huang *et al.*, 2007) show that over-expression of PrP in the muscle leads to a primary myopathy which correlates with high percentage of C1. This group suggests that the myopathy seen in these mice are due to activation of p53 signalling pathways which may be triggered by C1 over-production (Sunyach *et al.*, 2007, Guillot-Sestier

et al., 2009, Liang *et al.*, 2009). However, in these models it is impossible to distinguish whether the cell-toxicity is associated with an increase in full-length PrP^{C} or an increase in C1.

Over-expressing transgenic mice generally have shorter incubation periods compared to mice expressing normal levels of PrP. Transgenic mice expressing ovine PrP (KARQ and KVRQ) have similar incubation periods compared to ARQ and VRQ sheep after injection with SSBP/1 (Hunter, unpublished observations). Therefore these mice appear to be a good model for prion disease in the normal host. In terms of alpha cleavage KVRQ mice are not a good model for alpha cleavage as a lower percentage of C1 was seen in these mice compared to VRQ sheep (Chapter 4). Tg338 and KARQ are better models for alpha cleavage as they mimic what happens in the natural host (Chapter 4). It is difficult to compare KB6 and KB5 mice to the natural host as alpha cleavage levels in cattle were not investigated.

When immunoprecipitation was carried out on Tg338 spleen PrP^{C} could not be detected via Western blotting (Chapter 6). The spleen has been shown to be involved in neuroinvasion as removal of the spleen results in increased incubation periods in scrapie mouse models (Fraser & Dickinson, 1978, Kimberlin & Walker, 1989b). PrP^{Sc} also regularly accumulates in the spleen tissue during classical scrapie infection (van Keulen *et al*., 1996). DELFIA$^{®}$ measurements from the brains of Tg338 mice show that there is a 10 fold increase in the amount of PrP detected compared to KARQ and KVRQ mice that express normal levels of PrP. Assuming that PrP expression in the brain is 8-10 fold compared to wild type mice then the levels of PrP in the spleen should be equivalent to levels of PrP^{C} in a wild type mouse brain. This does not appear to be the case. In order to measure PrP protein levels in these tissues a DELFIA$^{®}$ assay could be carried although the sensitivity of the DEFFIA$^{®}$ assay may not be good enough for measuring PrP in peripheral tissues.

7.4 Final conclusions

The work presented here suggests that the PrP fragment C1 is not a significant determinant of TSE susceptibility. There may be some minor influence of PrP gene

polymorphisms on the production of the C1 fragment but this is not strong enough to modulate disease susceptibility. The C1 fragment does not appear to be associated with genetic variation of the PrP protein sequence as alpha cleavage occurred in mice expressing ovine, bovine and murine PrP sequence. PrP gene expression appears to be associated with the levels of C1 and possibly C2 production as PrP over-expressing transgenic mice containing the ovine, bovine and murine sequence show a higher percentage of C1 and C2.

The regulation of the C1 fragment is totally unknown but it appears from the data that PrP genetics is not associated with modulation of the C1 fragment. It is still unclear whether C1 takes part in the disease specific processes leading to PrP^{Sc} deposition and partial protease resistance. However, the percentage of C1 in uninfected tissue does not appear to be associated with differences in disease susceptibility and can not be correlated with incubation periods in sheep. It is possible that the percentage of C1 changes during infection which may be an important modulator of susceptibility. This remains to be investigated for sheep.

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Appendix i

Table I Metadata for all genotyped animals

Animal ID	Breed	Age (months)	Sex	136/154/171	141	112
014	CH	84	♀	ARR/ARR	L/L	M/M
017	CH	114	♀	ARR/ARR	L/L	M/M
022	CH	102	♀	ARR/ARR	L/L	M/M
030	CH	108	♀	ARR/ARR	L/L	M/M
051	CH	84	♀	ARR/ARR	L/L	M/M
75 x 49	CH	60	♂	ARR/ARR	L/L	M/M
050	CH	84	♀	AHQ/ARR	L/L	M/M
42 x 05	CH	36	♀	AHQ/ARR	L/L	M/M
47 x 29	CH	96	♀	AHQ/ARR	L/L	M/M
71 x 58	CH	72	♂	AHQ/ARR	L/L	M/M
81 x 80	CH	0	♀	AHQ/ARR	L/L	M/M
47 x 66	CH	48	♂	AHQ/ARQ	L/F	M/M
59 x 89	CH	108	♂	AHQ/ARQ	L/F	M/M
63 x 51	CH	72	♀	AHQ/ARQ	L/L	M/M
69 x 24	CH	84	♂	AHQ/ARQ	L/F	M/M
82 x 15	CH	0	♂	AHQ/ARQ	L/F	M/M
2018E	S	0-3	♂	ARQ/ARR	L/L	M/M
005D	S	3-6	♂	ARQ/ARR	L/L	M/M
2018E	S	0-3	♂	ARQ/ARR	L/L	M/M
005D	S	3-6	♂	ARQ/ARR	L/L	M/M
013D	S	3-6	♂	ARQ/ARR	L/L	M/M
021D	S	3-6	♂	ARQ/ARR	L/L	M/M
024D	S	3-6	♂	ARQ/ARR	L/L	M/M

43 x 39	CH	96	♀	ARQ/ARR	L/L	M/M
53 x 57	CH	120	♂	ARQ/ARR	L/F	M/M
61 x 13	CH	108	♀	ARQ/ARR	L/L	M/M
72 x 38	CH	72	♂	ARQ/ARR	L/L	M/M
74 x 00	CH	60	♂	ARQ/ARR	L/L	M/M
81 x 34	CH	0	♀	ARQ/ARR	L/L	M/M
J212	PD	12	♀	VRQ/VRQ	L/L	M/M
J225	PD	12	♂	VRQ/VRQ	L/L	M/M
J232	PD	12	♂	VRQ/VRQ	L/L	M/M
J236	PD	12	♀	VRQ/VRQ	L/L	M/M
J255	PD	12	♂	VRQ/VRQ	L/L	M/M
J368	CH	27	♀	VRQ/VRQ	L/L	M/M
J374	CH	27	♀	VRQ/VRQ	L/L	M/M
K295	CH	29	♀	VRQ/VRQ	L/L	M/M
K310	CH	29	♀	VRQ/VRQ	L/L	M/M
K320	CH	29	♀	VRQ/VRQ	L/L	M/M
H30	PD	24	♀	VRQ/ARQ	L/L	M/M
H41	PD	24	♀	VRQ/ARQ	L/L	M/M
H84	PD	24	♀	VRQ/ARQ	L/L	M/M
H50	PD	24	♀	VRQ/ARQ	L/L	M/M
Pg0109	NZCH	8	♂	VRQ/ARQ	L/L	M/M
Pg0437	NZCH	60	♀	VRQ/ARQ	L/L	M/M
Pg0490	NZCH	4	♂	VRQ/ARQ	L/L	M/M
Pg0675	NZCH	48	♀	VRQ/ARQ	L/F	M/M
Pg1226	NZCH	3	♂	VRQ/ARQ	L/F	M/M
6D	S	0	♂	ARQ/ARQ	L/L	T/T
97A	S	3-6	♂	ARQ/ARQ	L/L	M/M
2006E	S	0	♂	ARQ/ARQ	L/L	M/M

2016E	S	0	♀	ARQ/ARQ	L/L	M/M
B1227	S	3-6	♀	ARQ/ARQ	L/L	M/M
69 x 67	CH	84	♂	ARQ/ARQ	L/L	M/M
Pg0416	NZCH	36	♀	ARQ/ARQ	L/L	M/M
Pg0433	NZCH	24	♀	ARQ/ARQ	L/L	M/M
Pg0854	NZCH	36	♀	ARQ/ARQ	L/L	M/T

CH = Cheviot

CHNZ = New Zealand Cheviot

PD = Poll Dorset

S = Suffolk

♀ = Female

♂ = Male