

# Characterization of the Prion Protein in Relation to Normal Cellular Function and in Disease

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## Characterization of the Prion Protein in Relation to Normal Cellular Function and in Disease

### Abstract

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of rare and fatal neurodegenerative disorders that can affect both human and animals. Evidence indicates that the key event in prion disease pathogenesis is the conformational conversion of the normal cellular prion protein (PrP<sup>C</sup>) into an aggregated isoform called the scrapie prion protein (PrP<sup>Sc</sup>). The normal function of PrP<sup>C</sup> is still not known but the protein is essential for transmission of prion diseases. Defining the physiological activities of PrP<sup>C</sup> is crucial for understanding the normal function and pathogenesis of prion diseases. In this thesis, the proteolytic cleavages and shedding of PrP<sup>C</sup> were studied. PrP<sup>C</sup> was shown to be released from the cell by three different mechanisms. The first mechanism released a N-terminal fragment of PrP<sup>C</sup> by  $\alpha$ -cleavage, the second released the full length PrP<sup>C</sup> and a C-terminal fragment without GPI-anchor via an extreme C-terminal cleavage and a third mechanism released PrP<sup>C</sup> in association with exosomes. It was also shown that a deletion in the  $\alpha$ -cleavage site inhibited the  $\alpha$ -cleavage of PrP<sup>C</sup> and that the  $\alpha$ -cleavage likely took place at the cell surface. Metalloproteases have been suggested to be involved in the different cleavages. Here, it was shown that metalloproteases were involved in the cleavage of the extreme C-terminal end, but not in the  $\alpha$ -cleavage of PrP<sup>C</sup>.

Nor98/atypical scrapie was identified in Norway for the first time in 1998. Characterization of the molecular and genetic properties in two Swedish cases of Nor98 showed that unique proteinase K (PK)-resistant fragments are present in Nor98-affected sheep. The existence of two PK-resistant fragments that share overlapping regions suggests that at least two distinct PrP conformations are present in brain extracts from affected sheep.

Chronic wasting disease (CWD) affects cervids in North America. Here, it was shown that the PrP sequence of cervids in Scandinavia is similar to genotypes connected with CWD susceptibility in North American cervid species. Also, the European moose was shown to have a unique variation in codon 109 with 109K/Q and 109Q/Q.

*Keywords:* prion,  $\alpha$ -cleavage, shedding, exosomes, Nor98, CWD.

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To Chicko and my family.

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals (I-III) in the text:

- I **Wik L.**, Klingeborn M., Willander H. and Linné T. Separate mechanisms act concurrently to shed and release the prion protein from the cell.  
*Manuscript.*
- II Klingeborn M., **Wik L.**, Simonsson M., Renström L.H.M., Ottinger T. and Linné T. (2006). Characterization of proteinase K-resistant N- and C-terminally truncated PrP in Nor98 atypical scrapie. *J Gen Virol.* 87, 1751-60.
- III **Wik L.**, Mikko S., Klingeborn M., Steen M., Simonsson M. and Linné T. (2012). Polymorphisms and variants in the prion protein sequence of European Moose (*Alces alces*), reindeer (*Rangifer tarandus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) in Scandinavia. *Prion* 6(3), July/August/September.

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## Abbreviations

Three and one letter codes for the 20 naturally occurring amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V



## Other abbreviations used

A $\beta$	Amyloid $\beta$ -peptide
aa	Amino acid
Ab	Antibody
ADAM	A disintegrin and metalloproteinase
APP	Amyloid- $\beta$ precursor protein
BSE	Bovine spongiform encephalopathy
C1	C-terminal fragment of PrP with GPI-anchor, generated by the $\alpha$ -cleavage
C1-S	C-terminal fragment of PrP without GPI-anchor, generated by the $\alpha$ -cleavage and cleavage in the extreme C-terminal end
C2	C-terminal fragment of PrP generated by the $\beta$ -cleavage
CDS	Coding sequence
CJD	Creutzfeldt-Jakob disease
CWD	Chronic wasting disease
DNA	Deoxyribonucleic acid
Dpl	Doppel (prion-like protein)
ER	Endoplasmic reticulum
FFI	Fatal familial insomnia
FL	Full-length PrP
FL-S	Full-length PrP without GPI-anchor
FTIR	Fourier-transform infrared
GPI	Glycosylphosphatidylinositol
kDa	Kilodalton
mAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
MWS	Moose wasting syndrome
N1	N-terminal fragment of PrP generated by the $\alpha$ -cleavage
N2	N-terminal fragment of PrP generated by the $\beta$ -cleavage
ORF	Open reading frame
PK	Proteinase K
PMCA	Protein misfolding cyclic amplification
PNGaseF	Peptide: N-glycosidase F
<i>Prnd</i>	Dpl gene
<i>PRNP/Prnp</i>	PrP gene
PrP	Prion protein
PrP $\Delta$ <sup>121-123</sup>	Deletion mutant of the prion protein

PrP <sup>27-30</sup>	PK-resistant core of PrP <sup>Sc</sup>
PrP <sup>C</sup>	Cellular prion protein
PrP <sup>Sc</sup>	PK resistant, disease-associated form of PrP
PrP <sup>wt</sup>	Wild-type PrP
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
Sho	Shadoo (prion-like protein)
<i>Sprn</i>	Sho gene
TACE	Tumor necrosis factor- $\alpha$ -converting enzyme
TEM	Transmission electron microscopy
Tg mice	Transgenic mice
TSE	Transmissible spongiform encephalopathy

# 1 Introduction

## 1.1 Background

Prion diseases are a group of fatal neurodegenerative disorders that can affect both human and animals. Historically, the term “slow virus” was used to define the poorly defined disease-causing agent. Treatments known to inactivate viruses did not prevent infectivity (Alper *et al.*, 1966) and the disease was later found to be caused by an unconventional infectious agent. In 1997 Stanley B. Prusiner was awarded the Nobel Prize for his discovery of prions and the term prion was introduced to describe the proteinaceous infectious particle. Enrichment of infectious material resulted in the isolation of a partially protease resistant core of 27-30 kDa in size (Bolton *et al.*, 1984). Protein sequencing of this fraction allowed the identification of a cellular gene, the prion protein gene (*PRNP*) that encodes a protein known as the cellular prion protein ( $\text{PrP}^{\text{C}}$ ). As the infective protein component ( $\text{PrP}^{\text{Sc}}$ ) and the cellular prion protein share the same amino acid sequence, the distinctive properties of prions are determined by posttranslational modifications. In the early 1990s, prion diseases got public attention with the “Mad cow disease” or bovine spongiform encephalopathy (BSE).

## 1.2 Prion diseases

All prion diseases are collectively called transmissible spongiform encephalopathies (TSEs). Most TSEs are characterized by a long incubation period but once symptoms appear the disease progress rapidly leading to brain damage and death. Neuropathologically the disorders include deposition of amyloid plaques that produce a spongiform degeneration of the brain. The plaque deposits and regions of the brain affected vary depending on the prion strain and host. In addition, no inflammatory reactions are elicited upon infection.

### 1.2.1 Human prion diseases

Prion diseases in humans are relatively rare and can occur in a sporadic, genetic or transmissible form (Belay, 1999). Sporadic forms of prion diseases are evenly distributed across the world and arise due to spontaneous misfolding of the PrP or due to age related somatic mutations of the *PRNP*. Sporadic Creutzfeldt-Jakob disease (sCJD) accounts for around 85% of the prion diseases and are the most common human prion disease. Sequencing of the *PRNP* has provided information that codon 129 polymorphism leads to the protein containing either methionine (Met) or valine (Val) (Alperovitch *et al.*, 1999). Compelling evidence has shown that homozygosity for Met at codon 129 constitutes a risk for development of prion diseases. It has also been shown that methionine homozygotes are overrepresented among patients with sCJD.

All genetic or inherited cases of prion diseases include coding mutations in the *PRNP*. To date, over 40 different mutations in the *PRNP* have been shown (Colby & Prusiner, 2011). The genetic prion diseases have been classified as Gerstmann-Sträussler-Scheinker syndrome (GSS), familial (f) CJD and fatal familial insomnia (FFI). In GSS, the most common mutation is the proline (P) to leucine (L) substitution at codon 102 (P102L). In contrast to other inherited human prion diseases, GSS has unique neuropathologic features that consist of widespread PrP plaques. Previous studies have also shown the presence of a low molecular weight N- and C-terminally truncated PrP fragment that represent a molecular marker for this disorder (Parchi *et al.*, 1998). Familial CJD accounts for 5-10% of all CJD cases and has in general an earlier age of onset than other CJDs and the phenotype resembles that of sCJD (Meiner *et al.*, 1997). The most common form of fCJD is associated with mutations at codon 200 in the PrP sequence. A subtype of fCJD and FFI are both associated with a point mutation at codon 178 together with Met or Val in the polymorphic codon 129 of the PrP sequence (Gambetti *et al.*, 1995). The D178N, 129M allele segregates with FFI while the D178N, 129V allele segregates with fCJD. In FFI, the clinical course is dominated by progressive insomnia that is ultimately fatal (Manetto *et al.*, 1992) and the neuropathology of the disease shows marked neuronal loss, but spongiform changes or plaques are rarely demonstrated which has raised the question whether FFI should be classified as a TSE.

Infectious or transmissible forms of prion diseases include kuru, iatrogenic (i) CJD and variant (v) CJD. Kuru is a prion disease of humans that reached epidemic proportions in a defined population of Papua New Guinea (Johnson, 2005). The disease was transmitted during cannibalism in which members of

the tribe ate brain tissue of dead relatives due to religious reasons. Iatrogenic CJD is caused by exposure of prions due to injections of prion-contaminated growth hormones, implantation of dura mater grafts and through contaminated neurosurgical instruments (Johnson, 2005). In 1994 a novel form of a human prion disease named vCJD was described (Will *et al.*, 1996). Biochemical, neuropathologic and transmission studies have pointed to that vCJD resulted from prions being transmitted from cattle with bovine spongiform encephalopathy (BSE) to humans via consumption of contaminated meat products. From 1996 to 2001 the incidence of vCJD in the United Kingdom increased ([www.doh.gov.uk](http://www.doh.gov.uk)) but since then, the number of cases appears to have declined. More than 200 people have died of vCJD, mostly in Great Britain. A single case of vCJD heterozygous at codon 129 has been reported (Kaski *et al.*, 2009), all other vCJD-affected individuals have been Met homozygous at codon 129.

### 1.2.2 Animal prion diseases

#### *Scrapie*

Scrapie in sheep and goats was discovered over 200 years ago and was the first TSE to be identified (Plummer, 1946). The disease is characterized by intense pruritus and affected sheep rub and scrape until the wool is scratched off. Amino acid polymorphisms at codon 136 (A or V), 154 (A or H) and 171 (R or Q) have been reported to be associated with scrapie susceptibility (Belt *et al.*, 1995). The VRQ variant is considered the most susceptible while the ARR variant is considered most resistant to classical scrapie. In 2003, as a result of a decision in EU, a breeding program was set up to reduce the susceptible genotypes and improve the resistance by selectively breeding for the ARR genotype (EU, 2003). Experiments and epidemiological studies have shown that the spread of scrapie mainly occurs by horizontal transmission, either by prions being spread in the environment or direct transmission between sheep (Hoinville, 1996). In Sweden, there has only been one report of scrapie when two ewes in the same flock were diagnosed with the disease in 1986 (Elvander *et al.*, 1988). Western immunoblotting performed on brain homogenates from these two cases displayed the typical molecular characteristics of classical scrapie (Klingeborn *et al.*, 2006).

#### *BSE*

In 1986, the first case of BSE appeared in the UK and was followed by a massive epidemic with around one million infected animals (Nathanson *et al.*, 1997). The most accepted theory on the origin of BSE is that it was a

consequence of recycling infection by feeding cattle with meat and bone meal (MBM). MBM is a product of the rendering industry and it is primarily used as a protein supplement to cattle. In the late 1970s, the extraction method was changed in the rendering process and it is thought that this change allowed a larger fraction of scrapie prions from sheep or sporadically generated bovine prions to survive in the MBM, resulting in the widespread infection of cattle. In 1988, the use of animal-derived food supplement was banned in the UK and four years after the ban the cases began decreasing (Collee & Bradley, 1997a; Collee & Bradley, 1997b).

#### *Atypical BSE*

Atypical BSE that differ from classical BSE has been reported in different countries (Tranulis *et al.*, 2011). The atypical BSE agents have been classified into two types, the L- and H-types, relating to the lower and higher molecular weight of the unglycosylated band observed. After analysis of the coding sequence of the *PRNP* a genetic cause to the disease seemed unlikely as the coding sequence did not reveal any unusual features. In 2001, an active surveillance program was started in Europe to control, prevent and eradicate TSEs. BSE had never been diagnosed in Sweden until 2006 when one case was identified through the surveillance program. The case was a 12-year-old cow identified as an H-type BSE variant (Gavier-Widen *et al.*, 2008).

#### *Other animal prion diseases*

Transmissible mink encephalopathy (TME) is a TSE that affects ranched mink (Liberski *et al.*, 2009). Outbreaks seem to result from feeding minks with contaminated food containing prions. Feline spongiform encephalopathy (FSE) is a prion disease known to affect domestic and captive feline species (Sigurdson & Miller, 2003). The disease was first observed in the UK in 1990 and it is possible, although not proven, that animals got affected by eating contaminated bovine meat. Exotic ungulate encephalopathy (EUE) is a TSE of exotic zoo ruminants (Imran & Mahmood, 2011). The disease was overlapping with the BSE epidemic and the affected animals had been fed MBM.

#### *Nor98*

In 1998, an atypical form of scrapie was identified in Norwegian sheep for the first time and subsequently named Nor98 (Benestad *et al.*, 2003). The Nor98 cases were identified within the framework of the Norwegian national surveillance program for scrapie. According to collected data from 20 European countries, Nor98 has been shown to be a widespread disease (Fediaevsky *et al.*, 2008). Interestingly, affected animals seem to occur most

frequently in sheep with genotypes considered to confer a high resistance to scrapie infection (Klingeborn *et al.*, 2006; Gavier-Widen *et al.*, 2004; Benestad *et al.*, 2003). Nor98 has in addition to that, also been very rare in sheep carrying the VRQ genotype, which is considered to be the most susceptible genotype for classical scrapie. These findings are not in line with the breeding program set up for eliminating the most susceptible genotype variants to scrapie and may call for a reevaluation. Moum *et al.* (2005) investigated the *PRNP* in 38 Norwegian cases and showed that Nor98 was highly associated with polymorphisms at codons 141 and 154.

Nor98 cases differ from classical scrapie in several features. In classical scrapie for example, the predominance of the histopathological lesions is in the brain stem while Nor98 cases show pathology in the cerebral and cerebellar cortices (Benestad *et al.*, 2003). The clinical presentation and epidemiology of Nor98 also differ from classical scrapie (Benestad *et al.*, 2008). Nor98 cases are widely distributed geographically and normally only one or very few cases are seen in a flock and Nor98 cases are also generally older than classical scrapie cases. Major differences are also seen in detailed mapping of the PrP-fragments after proteinase K (PK) treatment and western blot. Classical scrapie displays a typical triplet pattern comprising the di- mono- and unglycosylated band migrating between 18 and 30 kDa (Klingeborn *et al.*, 2006). After PK treatment, Nor98 PrP-fragments display a distinct multiple band pattern with a characteristic fast migrating band about 11-12 (Arsac *et al.*, 2007; Nentwig *et al.*, 2007; Gretzschel *et al.*, 2006; Benestad *et al.*, 2003) or 7-8 kDa (Nentwig *et al.*, 2007; Klingeborn *et al.*, 2006). The disagreement regarding the estimated molecular mass of this prominent band of lower molecular mass is most likely due to different electrophoretic conditions used in the experiment or due to different PK conditions. By using a gradient SDS-PAGE Götte *et al.* (2011) showed that the lower molecular weight band involved two separate PK resistant fragments with molecular masses of approximately 5 and 8 kDa. Proteinase K resistant low molecular weight band containing ragged N- and C-termini has been described in other prion diseases such as GSS in humans (Tagliavini *et al.*, 1994). Nor98 could represent a valuable model for investigating pathogenesis of GSS and vice versa but it can only be speculated if both diseases involve similar pathomolecular mechanisms. Mutations described for GSS have not been detected in Nor98 and no mutations in the *PRNP* that can be correlated to Nor98 have been observed (Nentwig *et al.*, 2007). However, the distribution of the disease is widespread, only single cases in a flock are affected, Nor98 cases are generally older and there is a lack

of genetic evidence for the disease. These findings together might suggest that Nor98 is a spontaneous or a sporadic prion disease.

### *CWD*

Chronic wasting disease (CWD) is an emerging prion disease of deer, elk and moose. Of all mammalian prion diseases, CWD is likely the most efficiently transmitted and it is the only prion disease known to affect free-ranging animals. CWD was first described in 1967 and was reported as a TSE in 1978 on the basis of histopathology of the brain (Williams & Young, 1980). Presently, the disease occurs only in parts of USA and Canada but has been found in South Korea where it was introduced via import from Canada (Walsh & Miller, 2010; Kim *et al.*, 2005). PrP<sup>Sc</sup> has been shown to accumulate in skeletal muscle, blood, saliva, urine and feces, even in asymptomatic deer. How CWD is spread is not assured but hypotheses range from spread via direct contact, exposure through grazing in areas contaminated by prion-infected secretions or via decomposed carcasses. There are concerns that CWD prions will cross the species barrier into new hosts, including humans. Indirect studies of human susceptibility to CWD however, suggest that the risk is low. People have been exposed to CWD for many years since several million deer and elk hunters consume venison in the US and Canada. To study the human species barrier, Kong *et al.* inoculated transgenic mice expressing human PrP or elk PrP with elk CWD prions (Kong *et al.*, 2005). Elk PrP expressing mice developed disease only 118-142 days post-inoculation, while human PrP expressing mice did not develop any symptoms of disease after more than 657-756 days. Two other studies also reported that human PrP overexpressing mice were not susceptible to CWD prions (Sandberg *et al.*, 2010; Tamguney *et al.*, 2006).

Genetic studies of CWD have shown that there is a correlation between the PrP amino acid sequence and susceptibility to CWD infection. Polymorphisms at codon 95 (Q or H) and 96 (G or S) in the PrP sequence have been described in white-tailed deer (*Odocoileus virginianus*) (Johnson *et al.*, 2006). The Q95H and the G96S were linked to reduced susceptibility to CWD, but the disease has been identified in white-tailed deer of all genotypes. In a study of CWD in Rocky Mountain elk (*Cervus Canadensis nelsoni*) a polymorphism at codon 132 (methionine (M) or leucine (L)), corresponding to codon 129 in humans, were found (O'Rourke *et al.*, 1999). Homozygosity for M (132MM) was overrepresented in CWD-affected elk compared with elk heterozygous for M and L (132ML). However, a study in Wapiti (*Cervus elaphus nelsoni*) revealed that in the CWD affected cases, the three PrP codon 132 genotypes were

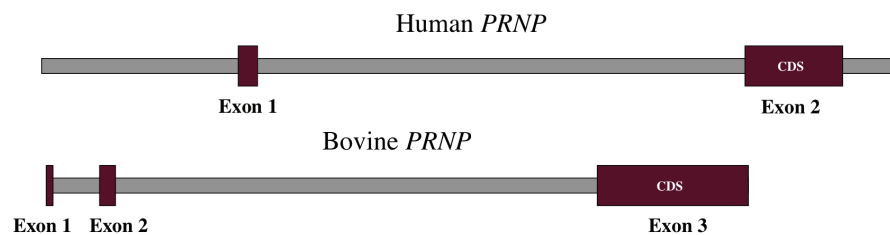


represented in proportion to their abundance in sampled populations and all three genotypes showed equivalent susceptibility to infection (Perucchini *et al.*, 2008). Mule deer (*Odocoileus hemionus*) carry a polymorphism at codon 225 (serine (S) or phenylalanine (F)) (Jewell *et al.*, 2005). The heterozygote 225SF animals were associated with longer incubation periods compared to animals homozygous for serine (225SS).

### 1.3 The cellular prion protein

#### 1.3.1 The *PRNP*

The prion protein gene (*PRNP* in humans and *Prnp* in mouse) is highly conserved across species. In mammals, the DNA sequence of the open reading frame (ORF) encoding PrP generally exhibits around 90% similarity (Schatzl *et al.*, 1995). In humans, the *PRNP* is a single copy gene positioned on chromosome 20. The human *PRNP* comprises two exons separated by a single intron with the entire ORF located in exon two (Fig. 1) (Puckett *et al.*, 1991). The *Prnp* of mice, sheep and cattle contains three exons with the protein coding sequence located in the third exon (Fig. 1) (Inoue *et al.*, 1997; Westaway *et al.*, 1994; Basler *et al.*, 1986). PrP mRNA is found primarily in neuronal cells (Kretzschmar *et al.*, 1986) but is also normally expressed in a variety of other tissues (Brown *et al.*, 1990). The mRNA is constitutively expressed in the brains of adult animals but highly regulated during development (Moser *et al.*, 1995; Mobley *et al.*, 1988).



*Figure 1.* Schematic presentation of the human and bovine prion protein gene. The coding sequence (CDS) is situated within exon two in the human *PRNP* and within exon three in the bovine *PRNP*. (The Gene accession nos. in the NCBI database is human NG\_009087 and bovine AC\_00017)

Two paralogs of the PrP, the Doppel protein and the Shadoo protein, have been identified. The doppel protein (Dpl) encoded by the *Dpl* gene (*Prnd*) is located downstream of the *Prnp* and is thought to have arisen by a duplication of the *Prnp* (Tranulis *et al.*, 2001; Moore *et al.*, 1999). Overall, the *Prnd*

protein product shares 25% identity and 50% similarity with the C-terminal domain of PrP. The similarities suggest that these two proteins may share some biological properties. At present, experimental data of Dpl is incomplete in regard to the possible involvement of Dpl in relation to PrP. Dpl is for example mainly expressed in testis but at very low levels in the brains and other tissues of adult mice. The difference in the expression pattern between Dpl and PrP indicated that Dpl is unlikely to be relevant to similar cellular roles as PrP. However, expression of Dpl in *Prnp* knockout mice resulted in a neurodegenerative phenotype that was rescued by the presence of PrP (Moore *et al.*, 2001). The PrP-like protein Shadoo (Sho) is another protein that show structural homology with PrP. Sho was detected via comparative gene analysis (Premzl *et al.*, 2003) and the Sho gene (*Sprn*) is located on chromosome 10 in humans, which are distinct from *Prnp* and *Prnd*. Although the function of Sho is unknown, its expression pattern overlaps that of PrP in the brain (Watts *et al.*, 2007).

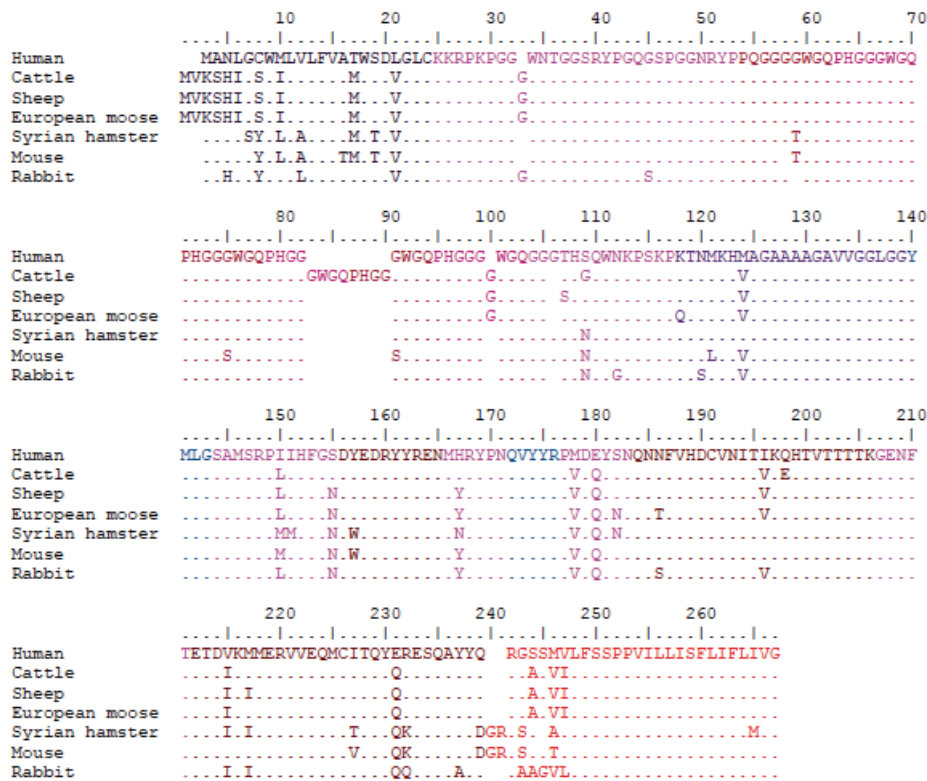
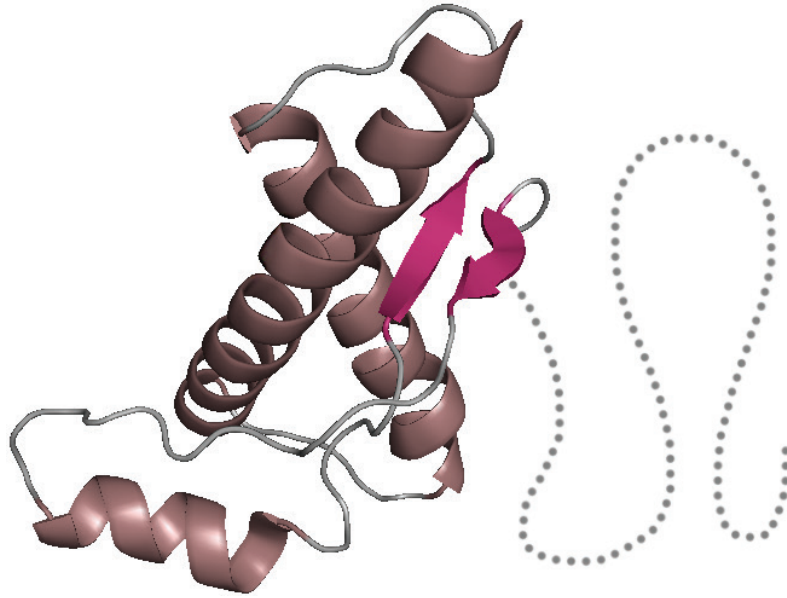


Figure 2. Alignment of the PrP sequence from human (P04156), cattle (P10279), sheep (NP001009481), European moose (AFF27617), Syrian hamster (P04273), mouse (AFF27617) and rabbit (AAC48679). Protein accession nos. according to the NCBI database. The multiple sequence alignment was done using ClustalW.

### 1.3.2 Cellular biology

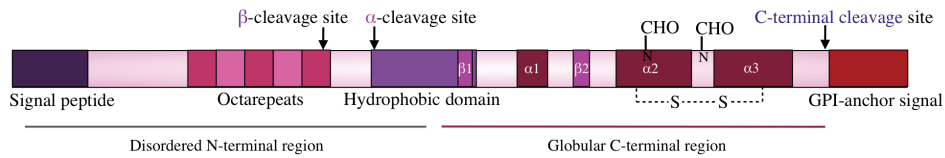
#### *Biosynthesis*

The mammalian PrP gene encodes a protein of approximately 250 aa that is highly conserved between species (Fig. 2). The mature protein can be divided in two regions, a flexible and unstructured N-terminal region and a C-terminal globular region arranged in three alpha helices interspersed with an antiparallel beta-sheet (Fig. 3).



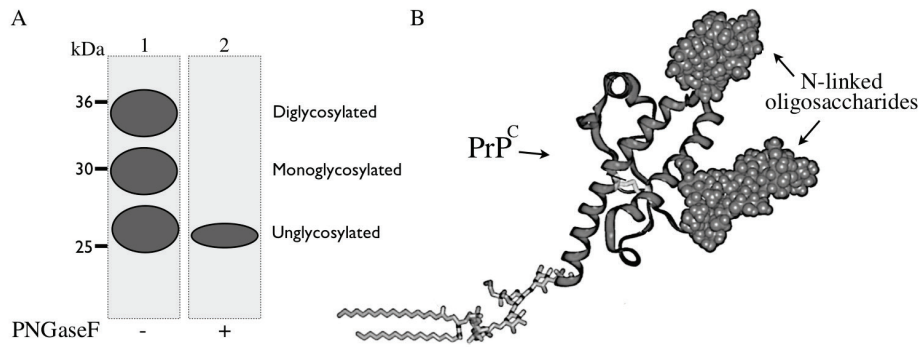
*Figure 3.* Cartoon of the three-dimensional structure the globular domain from the recombinant human PrP (residue 125 to 226), which contain three alpha-helices and a short anti-parallel beta sheet. The flexible N-terminal tail is represented by dots. The figure was prepared in the program MacPyMOL using the accession no. 1QLX from the RCSB protein data bank.

PrP<sup>C</sup> contains several distinct domains, including an N-terminal signal peptide, an octapeptide repeat (OR) region, a central hydrophobic region that is highly conserved, and a C-terminal hydrophobic portion that functions as a signal for addition of a glycosylphosphatidylinositol (GPI) anchor (Fig. 4). Human PrP consists of five copies of the octa repeat region (Fig. 2), in cattle however, six octa repeats are common but five repeats have also been shown (Nakamitsu *et al.*, 2006; Goldmann *et al.*, 1991).



*Figure 4.* Schematic illustration of the human PrP<sup>C</sup> primary translational product. The N-terminal signal peptide is cleaved off during the biosynthesis of the protein. The octarepeat region (OR) is believed to be essential for the binding of metal ions and the hydrophobic region is highlighted. PrP consists of an unstructured N-terminal region and a globular C-terminal domain. The consensus secondary structure in the C-terminal domain includes two  $\beta$ -strands and three  $\alpha$ -helices. The two glycosylation sites are shown with attached sugar groups represented as CHO. The disulfide bond is represented by the connection between  $\alpha$ -helix 2 and 3. The C-terminal signal sequence is cleaved of and replaced with a GPI-anchor in the mature protein. The cleavage sites are indicated by arrows.

PrP<sup>C</sup> is synthesized in the rough endoplasmic reticulum (ER). It is then passaged via the Golgi before it is transported to the cell surface where it is anchored to the plasma membrane via the GPI anchor. Throughout the biosynthesis, PrP<sup>C</sup> undergoes a number of post-translational modifications (Fig. 4). The N-terminal signal peptide is removed by peptidases, N-linked oligosaccharide chains are added at two asparagines, a disulfide bond is formed between two cystein residues, and a GPI anchor is attached (Haraguchi *et al.*, 1989; Stahl *et al.*, 1987). The glycosylation will result in di-, mono-, or unglycosylated forms with molecular weights of around 34, 28 and 25 kb, respectively (Russelakis-Carneiro *et al.*, 2002) (Fig. 5A). The N-linked oligosaccharide chains added initially in ER are modified in the Golgi to yield a complex chain that is resistant to endoglycosidase H (Caughey *et al.*, 1989) but can be cleaved off by Peptide-N-glycosidase F (PNGase F), giving rise to a 27 kDa full length PrP (Fig. 5A). The size of the complex oligosaccharides and the GPI anchor in relation to the polypeptide is substantial (Fig. 5B). Glycosylation of both asparagine sites yields over 400 different forms of PrP (Endo *et al.*, 1989). The physiological significance of these differences in glycosylation pattern remains unknown but observations have been made that different prion strains exhibit different patterns of glycosylation (Somerville *et al.*, 2005). It has also been suggested that the glycosylation is important and promotes correct folding of newly synthesized PrP as well as for trafficking and processing of PrP (Ermonval *et al.*, 2003).



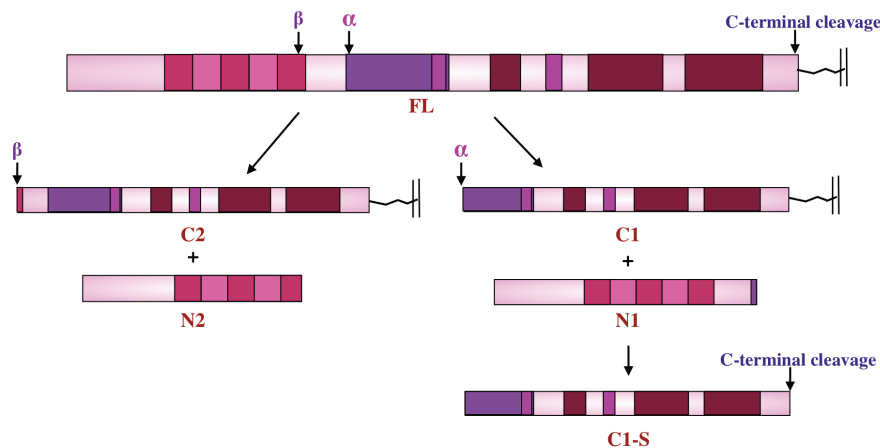
*Figure 5.* Glycosylation of PrP. A. Lane 1, schematic representation of the electrophoretic mobility of the three differently glycosylated moieties of PrP<sup>C</sup> (un-, mono-, and diglycosylated). Lane 2, the deglycosylated form of PrP<sup>C</sup> after treatment with PNGase F. B. Illustration of the globular C-terminal domain in human PrP indicating the position of N-linked oligosaccharides (Modified from Collinge J, *Neurol Neurosurg Psychiatry* 2005;76:906-919).

The GPI-anchored PrP<sup>C</sup> is localized predominantly in detergent-insoluble micro domains rich in cholesterol and sphingolipids in the plasma membrane, also named lipid rafts (Taylor & Hooper, 2006). It has been shown that PrP<sup>C</sup> after being exposed on the cell surface are subjected to endocytosis and recycled between the endocytic compartment and the plasma membrane (Zhao *et al.*, 2006; Harris, 1999; Vey *et al.*, 1996; Shyng *et al.*, 1994).

### 1.3.3 Post-translational cleavages

Our own work and that of others, with transfected cell lines as well as with brain tissue, have shown that PrP<sup>C</sup> is subjected to two post-translational cleavages (Zhao *et al.*, 2006; Chen *et al.*, 1995; Harris *et al.*, 1993; Stahl *et al.*, 1990) as illustrated in Fig. 6. One cleavage, referred to as the  $\alpha$ -cleavage, occurs between the alternative amino acid residues K<sub>110</sub>/H<sub>111</sub>/M<sub>112</sub> to yield a 11-kDa soluble N-terminal fragment called N1 and a 18-kDa C-terminal fragment called C1 that is still attached to the membrane via the GPI anchor. The  $\alpha$ -cleavage occurs within a hydrophobic segment that is highly conserved which underlines the importance of this processing (see Fig. 2, human PrP 112-138). The hydrophobic region is characterized as amyloidogenic and is thought to play a major role in the conformational change of PrP<sup>C</sup> to PrP<sup>Sc</sup> (Prusiner *et al.*, 1998). Our data indicate that the  $\alpha$ -cleavage takes place at the cell surface, but other cellular sites have also been suggested like endosomal/lysosomal

compartments and late compartments of the secretory pathway (Walmsley *et al.*, 2009; Tveit *et al.*, 2005; Taraboulos *et al.*, 1995; Shyng *et al.*, 1993).



*Figure 6.* Processing of the full length (FL) PrP. The proteolytic  $\alpha$ -cleavage within the hydrophobic region results in a C-terminal fragment (C1) with intact GPI-anchor and a N-terminal fragment (N1). Cleavage in the extreme C-terminal end results in the full length PrP without GPI-anchor and a C-terminal fragment (C1-S) without GPI-anchor. The alternative  $\beta$ -cleavage generates a C-terminal fragment (C2) (with intact neurotoxic core) and a N-terminal fragment (N2).

Certain PrP<sup>C</sup> mutations close to or in the  $\alpha$ -cleavage site have been analyzed. Mutations in the central hydrophobic core, two aa C-terminal to the  $\alpha$ -cleavage site altered the resistance to proteases, the processing and the  $\alpha$ -cleavage of PrP<sup>C</sup> (Wegner *et al.*, 2002). Deletion of the three aa in the  $\alpha$ -cleavage site changed the processing of PrP and partially hindered the  $\alpha$ -cleavage (Wik, 2012). Another experiment where the  $\alpha$ -cleavage site was removed by deleting three or ten aa directly after the  $\alpha$ -cleavage site also affected the  $\alpha$ -cleavage (Mange *et al.*, 2004). When expressed in Tg mice, PrP mutants bearing large deletions around the  $\alpha$ -cleavage site induced a rapidly progressive, lethal phenotype and showed a complete inhibition of the  $\alpha$ -cleavage (Baumann *et al.*, 2007; Li *et al.*, 2007). These results were in accordance with the observation in a cell line where a large deletion of the hydrophobic part surrounding the  $\alpha$ -cleavage site prevented the  $\alpha$ -cleavage (Sakudo *et al.*, 2005). Point mutations in the cleavage site and replacement of the three aa in the  $\alpha$ -cleavage site with three alanines did not affect the  $\alpha$ -cleavage (Tveit *et al.*, 2005). In a recent study made by Oliveira-Martins *et al.*

(2010) they used a number of PrP<sup>C</sup> mutants to examine the tolerance of the  $\alpha$ -cleavage. The  $\alpha$ -cleavage was shown to be remarkably tolerant to variations in the sequence surrounding the  $\alpha$ -cleavage site. The  $\alpha$ -cleavage was independent of the precise sequence in the cleavage site, and was largely independent of the charges and hydrophobicity surrounding the cleavage site. Taken together, these results imply that  $\alpha$ -cleavage is performed by a  $\alpha$ -PrPase that has low sequence specificity and is dependent on the size of the region surrounding the  $\alpha$ -cleavage site.

A number of proteases have been implicated in the  $\alpha$ -cleavage process. It has been suggested that ADAM10 and TACE (ADAM17) are involved in the  $\alpha$ -cleavage of PrP<sup>C</sup> (Vincent *et al.*, 2001; Jimenez-Huete *et al.*, 1998), but evidence against the involvement of these metalloproteases in  $\alpha$ -cleavage has also been reported (Endres *et al.*, 2009; Vincent *et al.*, 2001; Jimenez-Huete *et al.*, 1998). Altmeyden *et al.* (2011) used neuro-specific ADAM10 knockout mice to show that ADAM10 was not responsible for the  $\alpha$ -cleavage. In fact, the C1- and N1 signals were even stronger in these mice but in the experiment, the amount of PrP<sup>C</sup> rather than the presence of ADAM10 correlated with the appearance of C1. Recently, ADAM8 was shown to directly cleave PrP *in vitro* and the  $\alpha$ -cleavage was greatly diminished in skeletal muscle of ADAM8 knockout mice (Liang *et al.*, 2012). In conclusion, the  $\alpha$ -PrPase still needs to be identified.

The function of the  $\alpha$ -cleavage is still not known but it could be a way to deplete the protein of its potential pathogenicity as the cleavage disrupts the neurotoxic domain within the PrP. The two products of  $\alpha$ -cleavage, C1 and N1 have been shown to be biologically active. The N1 fragment has a neuroprotective function by modulating the p53 pathway and the N-terminal domain has been shown to be crucial for the neuroprotective function of PrP (Turnbaugh *et al.*, 2011; Guillot-Sestier *et al.*, 2009). It has also been suggested that the N1 fragment could interfere with A $\beta$ -associated toxicity (Guillot-Sestier *et al.*, 2012). Transgenic mice expressing the C1-fragment, Tg(C1), have been shown to not produce any neurological disease or protease resistant PrP, not even when challenged by scrapie inoculation (Westergard *et al.*, 2011). Interestingly, the Tg(C1) mice expressing C1 together with wild-type PrP had a dramatically delayed time course to become ill compared with wild-type mice. These results demonstrate that the C1-fragment can act as an inhibitor of PrP<sup>Sc</sup> formation.

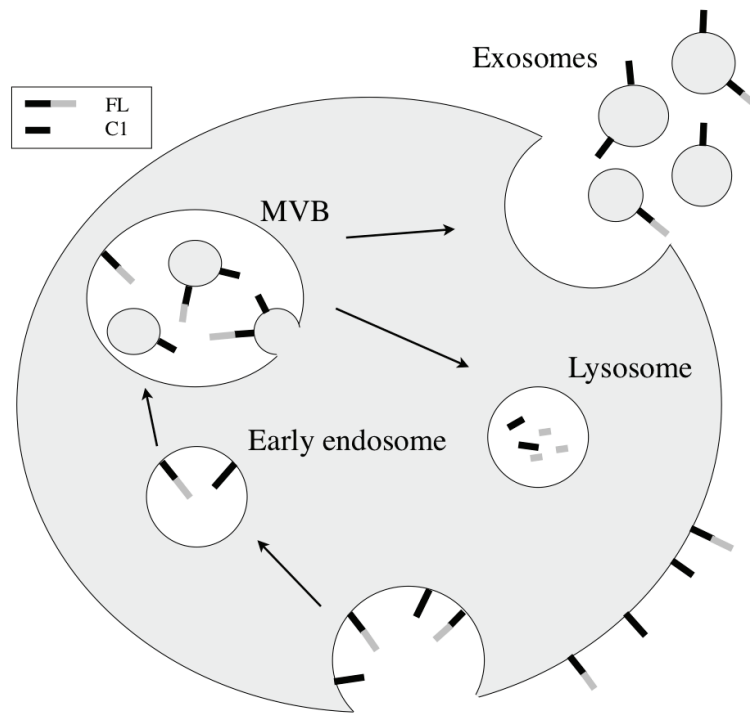
The second cleavage occurs at the extreme C-terminal end of PrP<sup>C</sup> close to the GPI-anchor (Fig. 6). Analysis has revealed that the cleavage takes place between Gly228 and Arg229, three residues away from the GPI-anchor attachment site (Taylor *et al.*, 2009; Zhao *et al.*, 2006; Stahl *et al.*, 1990). The cleavage results in shedding of full-length (FL) PrP and C1 fragment into the extracellular medium (Harris *et al.*, 1993). It has also been shown that these cleaved fragments are present *in vivo* in human cerebrospinal fluid (Borchelt *et al.*, 1993; Harris *et al.*, 1993). Recently, it has been shown that ADAM9 in the presence of ADAM10 is responsible for ectodomain shedding of PrP<sup>C</sup> and that ADAM10 directly can cleave PrP close to the GPI-anchor (Altmeppen *et al.*, 2011; Taylor *et al.*, 2009). In addition, lack of ADAM10 activity results in increased amounts of PrP<sup>C</sup> due to its retention inside the cell.

Another cleavage called the  $\beta$ -cleavage occurs between aa residues 89/90 in humans, generating a soluble N2 fragment and a GPI-anchored C2 fragment (Mange *et al.*, 2004; Jimenez-Huete *et al.*, 1998; Chen *et al.*, 1995), illustrated in Fig. 6. The  $\beta$ -cleavage preserves the cytotoxic and fibrillogenic core that is critical for conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. The C2 fragment corresponds to the protease resistant core or PrP<sup>Sc</sup> and the pathogenic isoform has been shown to be strain specific (Nicot & Baron, 2010). In a recent study it was found that production of the C2 fragment is strongly cell- and tissue dependent (Dron *et al.*, 2010). The  $\beta$ -cleavage is mediated by reactive oxygen species (ROS) as has been shown by increased  $\beta$ -cleavage when exposing cells expressing PrP<sup>C</sup> to H<sub>2</sub>O<sub>2</sub> in the presence of Cu<sup>2+</sup> (Watt & Hooper, 2005; Watt *et al.*, 2005; McMahon *et al.*, 2001). Observations also indicate that lack of  $\beta$ -cleavage correlates with an increased sensitivity to oxidative stress in cells and could implicate that  $\beta$ -cleavage as an early step in the cellular response to oxidative stress.

#### 1.3.4 Exosomes

Shedding of PrP<sup>C</sup> outside the cell is mainly driven by the  $\alpha$ -cleavage releasing the N1 fragment and the extreme C-terminal cleavage releasing FL PrP and C1 fragment to the medium. In addition to this, it has been shown that PrP<sup>C</sup> and PrP<sup>Sc</sup> are released in association with exosomes (Vella *et al.*, 2007; Leblanc *et al.*, 2006; Fevrier *et al.*, 2004).





*Figure 7.* Simplified schematic illustration of the sorting of PrP in multivesicular bodies (MBVs) and exosomes. PrP is internalized into early endosomes and inward budding of endosomes into their lumen forms internal vesicles. MBVs can fuse with the plasma membrane and the internal vesicles are then released extracellularly as exosomes. Alternatively, MBVs can fuse with lysosomes. Full length (FL) PrP and cleaved PrP into C-terminal fragments (C1) are indicated.

Exosomes are small membrane vesicles, around 50- to 100-nm in diameter, formed intracellularly by invagination of the membrane of endocytic compartments, leading to vesicle-containing endosomes called multivesicular bodies (MBVs) (Stoorvogel *et al.*, 2002) illustrated in Fig. 7. Exosome secretion into the extracellular matrix occurs upon fusion of MBVs with the cell membrane thus releasing their internal exosomes outside the cell. A wide variety of cultured cell types have been reported to secrete exosomes (Fevrier *et al.*, 2005). A large number of proteins and lipids are enriched in exosomes and among them are Tsg101 and Alix, components of the endosomal sorting complexes. Other cytosolic proteins common in exosomes are chaperone proteins like Hsp90 and Hsc70, cytoskeletal proteins (actin, tubulin, moesin) and annexins (Fevrier *et al.*, 2005). There are several suggested functions for

exosomes and some examples are roles in cell-to-cell signaling, removal of unwanted molecules and transfer of proteins and lipids between cells.

In three separate studies using different cell systems, it was demonstrated that exosomes from prion-infected cells could initiate prion propagation in recipient cells (Alais *et al.*, 2008; Vella *et al.*, 2007; Fevrier *et al.*, 2004). Exosomes from infected cells could also produce prion disease when inoculated into mice (Vella *et al.*, 2007; Fevrier *et al.*, 2004). Moreover, it has been shown that exosomes containing PrP<sup>C</sup> can be isolated from cerebrospinal fluid (Vella *et al.*, 2008). After inward budding of the plasma membrane, as the initial step in the constitutive cycling between the plasma membrane and endocytic compartments, PrP retains its GPI-anchor. Consistent with this, it was shown that exosome associated PrP is GPI-anchored (Vella *et al.*, 2007). These data suggests that exosomes could be a way to spread prions and are interesting particles in prion infectivity.

### 1.3.5 Function of PrP<sup>C</sup>

Although PrP was discovered almost thirty years ago and has been extensively studied, its physiological role is still not ensured. Establishing the normal function of PrP could contribute to the understanding of prion diseases and the mechanisms behind them. Several lines of PrP-knockout (PrP<sup>0/0</sup>) mice have been created and investigated (Manson *et al.*, 1994; Bueler *et al.*, 1993) and is one way to clarify the functions of PrP<sup>C</sup>. Mice lacking PrP<sup>C</sup> do not show any obvious phenotype and they develop normally and have a normal lifespan. The only marked phenotype of PrP<sup>0/0</sup> mice is their resistance to prion infection. However, some of these PrP<sup>0/0</sup> mice have been reported to have small phenotypic abnormalities like sleep disturbances and altered circadian rhythms (Steele *et al.*, 2007; Tobler *et al.*, 1996).

Another way to gain insight into the function of PrP<sup>C</sup> is to identify proteins that interact with and bind to PrP<sup>C</sup>. Identified interactors are for example molecules involved in adhesion (Santuccione *et al.*, 2005; Gauczynski *et al.*, 2001; Schmitt-Ulms *et al.*, 2001), trafficking (Parkyn *et al.*, 2008) and ion channel activity (Kleene *et al.*, 2007).

Several functions for PrP<sup>C</sup> have been proposed over the years. Some examples are involvement in long-term memory (Shorter & Lindquist, 2005; Nishida *et al.*, 1997), anti-apoptotic activities (Bounhar *et al.*, 2001), cell communication via exosomes (Fevrier *et al.*, 2004) signal transduction

(Mouillet-Richard *et al.*, 2000) and protection of cells from oxidative stress (Milhavet & Lehmann, 2002). Each of the octarepeat-residues in PrP<sup>C</sup> binds Cu<sup>2+</sup> and this suggests that PrP<sup>C</sup> could be involved in copper metabolism (Jackson *et al.*, 2001; Brown *et al.*, 1997).

## 1.4 Prions, PrP<sup>Sc</sup>

### 1.4.1 Cellular biology

PrP<sup>Sc</sup>, the abnormal isoform or PrP was suggested to be the constituent of the prion agent in the beginning of the 1980s. PrP<sup>Sc</sup> is detected by limited protease digestion that results in a protease resistant molecule of around 142 amino acids referred to as PrP27-30. The protease resistant core can form aggregates and amyloids (Prusiner *et al.*, 1983) but it has been demonstrated that the amyloid properties of PrP<sup>Sc</sup> are not an obligatory feature of prion diseases (Wille *et al.*, 2000). It has also been shown that not all PrP<sup>Sc</sup> molecules are resistant to protease digestion (Gambetti *et al.*, 2008) and the protease resistant fragments can have different resistant cores giving rise to shorter fragments when treated with proteases. Highly purified PrP<sup>Sc</sup> is also recognized by its insolubility in detergents and by infectivity in animal bioassays.

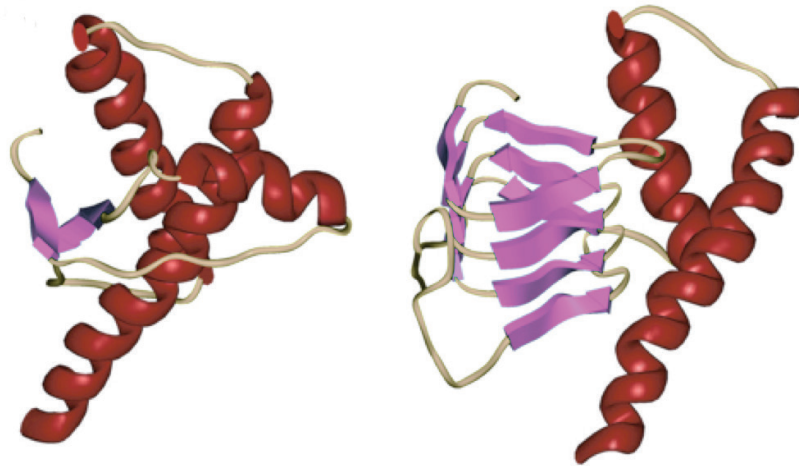


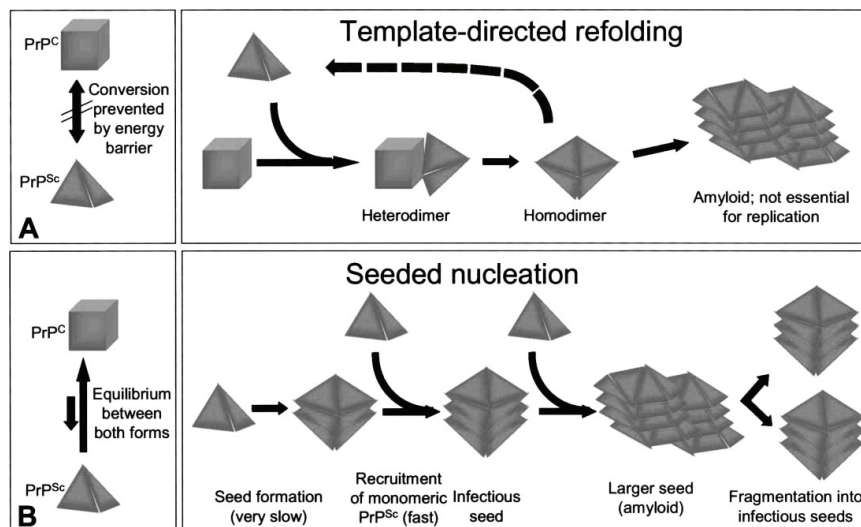
Figure 8. Model of the conversion of PrP<sup>C</sup> (left) to PrP<sup>Sc</sup> (right). The PrP<sup>Sc</sup> are suggested to contain a parallel left-handed  $\beta$ -helical structure. (Modified from Wille H *et. al.* PNAS 2002 Mar 19;99(6):3563-8)

The key event in the pathogenesis of prion diseases is the conformational change of the PrP<sup>C</sup> to PrP<sup>Sc</sup>, which involves drastical changes in the configuration and biochemical properties of the protein. Using Fourier-

transform infrared (FTIR) spectroscopy it was demonstrated that PrP<sup>C</sup> is composed of 47%  $\alpha$ -helix and only 3%  $\beta$ -sheet (Pan *et al.*, 1993). In contrast, the PrP<sup>Sc</sup> consisted of 43%  $\beta$ -sheet and 30%  $\alpha$ -helix (Fig. 8). Because PrP<sup>Sc</sup> is insoluble and forms aggregates it has been hard to determine the atomic structure of PrP<sup>Sc</sup>. However, through crystallographic studies together with computational modeling insight have been gained into the atomic structure of PrP<sup>Sc</sup> and a parallel left-handed  $\beta$ -helical fold has been suggested (Govaerts *et al.*, 2004; Wille *et al.*, 2002), illustrated in Fig.8.

#### 1.4.2 Propagation and amplification assays

The precise mechanism by which infectious PrP<sup>Sc</sup> induce conformational changes in PrP<sup>C</sup> is not known. Two different models have been proposed to explain the self-propagating conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> (Fig. 9).



*Figure 9.* Model for the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>. A. The refolding model predicts that PrP<sup>C</sup> refolds under the influence of an exogenous PrP<sup>Sc</sup> molecule. B. The seeding model postulates that PrP<sup>C</sup> and PrP<sup>Sc</sup> are in equilibrium with PrP<sup>C</sup> strongly favored. Once the seed is present, polymerization can efficiently take place. Fragmentation of the polymer increases the number of ends for the recruitment of monomers. (From Aguzzi A, Polymenidou M, Cell 2004; Jan 23;116(2):313-27)

The template-assisted model (also called the refolding model) predicts that the conversion of PrP<sup>C</sup> is induced by exogenous formed PrP<sup>Sc</sup> and is possibly catalyzed by an enzyme or a chaperone (Prusiner, 1991). A high energy barrier may prevent spontaneous conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup>. The nucleated-

polymerization model (also called the seeded nucleation model) postulates that the isoforms are in equilibrium and reversible but with the PrP<sup>Sc</sup> monomer much less stable and PrP<sup>C</sup> strongly favored (Jarrett & Lansbury, 1993). Once the nucleus has formed, monomeric PrP<sup>C</sup> adopt the conformation of PrP<sup>Sc</sup> and can efficiently add to the nucleus.

#### *Conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> in cell-free systems*

*In vitro* conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> was initially demonstrated in a cell-free system in which radioactively labeled PrP<sup>C</sup> became partially protease resistant when it was incubated with brain-derived PrP<sup>Sc</sup> (Kocisko *et al.*, 1994). In 2001, Soto and colleagues described a new type of cell-free conversion called protein-misfolding cyclic amplification (PMCA) (Saborio *et al.*, 2001). In a PMCA reaction, normal brain extracts are used as a source of PrP<sup>C</sup> and mixed with small quantities of brain homogenate from TSE-infected animals (or partially purified PrP<sup>Sc</sup>). The amplification process then involves repeated cycles of incubation and sonication resulting in highly efficient amplification of the PrP<sup>Sc</sup> conformation. Diluting the product in one reaction with fresh PrP<sup>C</sup> can increase the sensitivity and this also totally eliminates the original input of PrP<sup>Sc</sup>. This method is highly sensitive for detection of PrP<sup>Sc</sup> in tissues, blood and environmental samples.

#### 1.4.3 Prion strains and species barrier

One of the remarkable features of prion diseases is the existence of distinct prion strains. The strains consist of isolates that can generate distinct phenotypes in identical hosts and the clinical and biochemical properties can be conserved through several passages in rodent models (Pattison & Millson, 1961). To explain this, it was proposed that prion strains are a result from the conformational variability of PrP (Prusiner, 1991). Analysis of protease-resistant prion protein that was isolated from infected animals with different phenotypes showed distinct biochemical variations (Legname *et al.*, 2006; Telling *et al.*, 1996; Bessen & Marsh, 1992a). The variations included different glycosylation patterns, electrophoretic mobility of the protease resistant fragments, conformational stability and extent of protease resistance. The different prion strains can also be distinguished by distinct incubation time and patterns of neuropathology. The strains cannot be encoded by the primary structure in the PrP as they can be serially propagated in mice with identical PrP coding sequence (Fig. 10). The strains can then be re-isolated in mice after passage in other species with different PrP primary structure.

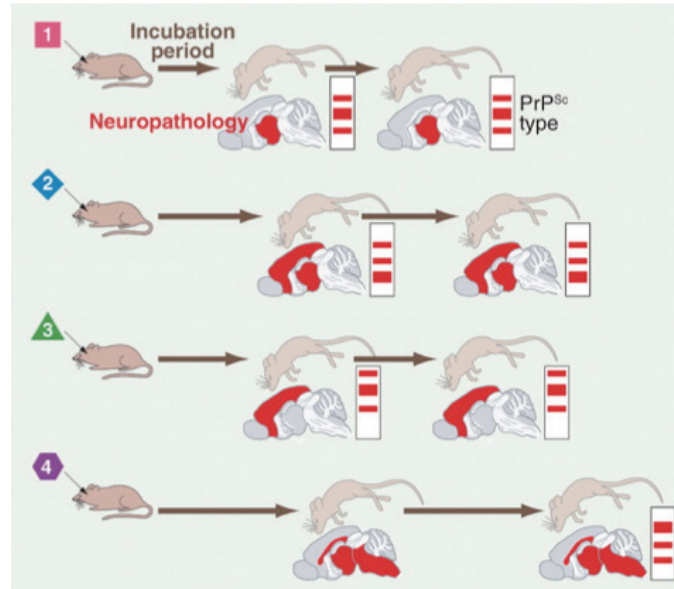


Figure 10. Propagation of prion strains. Standard criteria for characterizing and differentiating strains include the incubation time (length of arrow), neuropathology (marked brain area) and electrophoretic mobility after proteolysis (fragment pattern in Western blot, vertical bars). Distinct biochemical and biological properties of a single strain is retained after serial passage in the same host. (Modified from J Collinge, A R Clark, Science 2007; 318:930-936)

Two examples of biologically defined prion strains are the hyper (HY) and drowsy (DY) strains of TME in Syrian hamsters (Bessen & Marsh, 1992b). After serial passages from the same inoculation of infectious material into Syrian hamsters the incubation period became stable in two different groups with different clinical signs and histopathological changes. Also, it was found that the PK-resistant core of PrP<sup>Sc</sup> from DY and HY differed in their electrophoretic mobility, implying that the two strains had different conformations. Similarly, distinct human PrP<sup>Sc</sup> have been identified that are associated with different biochemical and pathological properties of CJD (Korth *et al.*, 2003; Collinge *et al.*, 1996). Recently, it was also demonstrated that recombinant PrP refolded into different amyloid conformations gave rise to primary prion strains, that, when inoculated into mice resulted in different strains (Colby *et al.*, 2009). The incubation periods in mice were dependent on the conformational stability of the primary recombinant PrP amyloid.

The finding that a particular strain can be transmitted between two species with different PrP sequence, without changing its strain-specific properties, implies that the same conformation can be imposed on different PrP sequence. The mechanisms behind this occurrence are not known and add to the difficulty in assessing the risk of interspecies spread of different prion strains in sheep, cervids and cattle.

#### 1.4.4 The protein-only hypothesis

The idea that the prion is the sole component responsible for prion diseases has been controversial since the “protein-only” hypothesis was suggested many years ago (Griffith, 1967). A number of lines of evidence support this hypothesis. For example, Alper *et al.* (1967) demonstrated that procedures that destroy nucleic acids, such as high doses of ionizing radiation and UV-light, did not inactivate the infectious material. Also, the PrP<sup>Sc</sup> and scrapie infectivity co-purify and highly purified preparations of PrP<sup>Sc</sup> can transmit the disease (Prusiner *et al.*, 1984; Bolton *et al.*, 1982). Moreover, mutations in the *PRNP* has been genetically linked to inherited prion diseases and overexpression of mutant *Prnp* in mice produces TSEs (Hsiao *et al.*, 1990). Strong evidence supporting the “protein-only” hypothesis came from the demonstration that *Prnp* knockout mice were resistant to scrapie infection. The cell-free conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> and the PMCA technique also added evidence to the hypothesis. Refolding PrP into an infectious conformation *in vitro* has been considered to be the final proof of the “protein-only” hypothesis. In 2004, synthetic prions were produced *in vitro* and injection of this material into Tg mice induced a TSE (Legname *et al.*, 2004). Unfortunately, the recombinantly produced prions were only transmissible to Tg mice overexpressing PrP and not to wt mice. However, in a recent work a recombinant prion was created that caused prion disease approximately 130 days after inoculation in wild-type mice (Wang *et al.*, 2010).

Despite compelling evidence for the prion hypothesis, several questions of prion infectivity remain. One question is whether the replication of prions requires a cellular co-factor. If a co-factor is needed it could for example act as a catalyst for conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup>, help stabilizing the conformation of PrP<sup>Sc</sup> or participate in the fragmentation of PrP<sup>Sc</sup>. A number of experimental evidence exists that demonstrates the involvement of a co-factor. For example, purified PrP<sup>C</sup> was not converted in PMCA studies when mixed with highly purified PrP<sup>Sc</sup> (Saborio *et al.*, 1999) but conversion was restored when

complete brain homogenate (normal) was added which suggest that factors present in brain homogenate are essential for conversion. Studies have also shown that natural or synthetic RNA can facilitate propagation of PrP<sup>Sc</sup> in hamster (Deleault *et al.*, 2003) but do not promote the propagation of mouse and vole PrP<sup>Sc</sup> molecules (Deleault *et al.*, 2010).



## 2 Present investigation

### 2.1 Aim of the thesis

The overall aim of this thesis was to study the function of the cellular prion protein and to characterize the genetic and molecular features of two different prion diseases.

In particular the studies were aimed to:

- Investigate the mechanisms for shedding and proteolytic cleavages of the cellular prion protein, in order to gain further insights into the function of the normal prion protein and pathogenesis of prion diseases (**paper I**).
- Characterize the molecular properties of Nor98 atypical scrapie in order to compare the disease to classical scrapie and to elucidate if the disease was associated with a specific genetic background (**paper II**).
- Examine the presence of sequence variants in the prion protein from deer and elk in Scandinavia and to compare those with the sequence variants associated with chronic wasting disease in North American cervids (**paper III**).

## 2.2 Results and discussion

This section summarizes the main results of Papers I-III that constitute this thesis, together with additional discussions and unpublished data.

### 2.2.1 Paper I: Separate mechanisms act concurrently to shed and release the prion protein from the cell.

The ability of a protein to possess infectious information has major biological implications. Therefore, defining the mechanisms behind the molecular background of prion propagation is important and will require knowledge of the structure, processing, transport and physiological functions of the PrP<sup>C</sup>. Factors influencing the two different cleavages of PrP<sup>C</sup> is a critical issue since the cleavages and its products are likely to have important biological functions and are probably also involved in the pathogenesis of PrP<sup>Sc</sup>. Another major question is the spread and transport of both PrP<sup>C</sup> and PrP<sup>Sc</sup> between cells.

It has earlier been shown that PrP<sup>C</sup> is post-translationally processed, and in normal brain, PrP<sup>C</sup> can be found both as a full-length (FL) PrP and processed to a C-terminal PrP fragment (C1) and a N-terminal fragment (N1). Previous studies have shown that PrP<sup>C</sup> is cleaved between residues K<sub>110</sub>H<sub>111</sub>M<sub>112</sub> (human numbering) during its normal processing (Zhao *et al.*, 2006; Chen *et al.*, 1995). This cleavage is referred to as the  $\alpha$ -cleavage. The pathogenic form, PrP<sup>Sc</sup>, seems to have an intact  $\alpha$ -cleavage site and is cleaved at an alternative residue around 20 aa N-terminal to the  $\alpha$ -cleavage site. One hypothesis is that the  $\alpha$ -cleavage disrupts the region necessary for the conformational change of PrP<sup>C</sup> to PrP<sup>Sc</sup> and thereby prevents the disease. Whereas the majority of PrP<sup>C</sup> is bound to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, secreted forms of the protein have been identified. PrP<sup>C</sup> molecules have also been shown to cycle between the plasma membrane and endocytic compartments inside the cell (Harris, 1999).

Studies of PrP *in vitro* have added much knowledge to our understanding of PrP<sup>C</sup>, PrP<sup>Sc</sup> and transmission of prions. In paper I, we used an eukaryotic expression system, based on the semliki forest virus (SFV) vector together with mammalian cells, which has been proven to be an efficient protein expression system. The SFV expression vector was originally described by Kaariainen *et al.* (1975). Briefly, the SFV genome is a single-stranded, positive RNA (i.e. functions as a mRNA), which encodes both structural, and nonstructural viral proteins. The vector pSFV1 is based on a full-length cDNA clone of SFV in which the coding region of the structural genes has been deleted (the 26S

promoter is retained) but the nonstructural coding region is preserved which is required for the replicase complex. The gene of interest, in this case the ORF of the *PRNP*, is cloned in the place of the structural genes. In the absence of the genes coding for viral coat proteins, viral RNA cannot be packed into infectious viral particles. The mRNA is transcribed *in vitro* and transfected into cells where it serves as template for the synthesis of, in our case, the different PrP constructs. The cell lysate and the cell medium are then analyzed for the presence of PrP.

In paper I, the main focus was to analyze the different PrP<sup>C</sup> fragments released from the cell into the extracellular medium. By defining the given N- and C-terminal fragments generated in the medium, the different cleavage events of PrP<sup>C</sup> taking place at the cell membrane will be reflected. The results presented in paper I suggest that PrP<sup>C</sup> is concurrently shed outside the cell via three separate mechanisms. The first mechanism releases the N1 fragment via the  $\alpha$ -cleavage and the second mechanism releases the FL-S and the C1-S (soluble fragments lacking the GPI-anchor) by proteolytic cleavage in the extreme C-terminal. The third mechanism is a slow process that releases a GPI-anchored fraction of PrP<sup>C</sup> in association with exosomes.

In order to analyze the  $\alpha$ -cleavage, the mutant PrP <sup>$\Delta$ 121-123</sup> was created by deleting three aa (corresponding to the amino acids KHV) encompassing the  $\alpha$ -cleavage site. When the  $\alpha$ -cleavage site was deleted, the accumulated N1 fragment in the cell medium was decreased by about 50%. When the cell lysate was analyzed, a decreased amount of the C1 fragment was found in the PrP <sup>$\Delta$ 121-123</sup> expressing cells. Further, in our experimental system used in paper I, the high level of expression enabled a short pulse-labeling approach to determine the time-course for the processing of PrP<sup>C</sup>. In the cell lysate a 45% decrease in the rate of the  $\alpha$ -cleavage was seen in the PrP <sup>$\Delta$ 121-123</sup> expressing cells. Together, these results indicate that deletion of the three aa in the  $\alpha$ -cleavage site hindered the  $\alpha$ -cleavage and also that the proteases involved in the  $\alpha$ -cleavage process possess sequence specificity. It has previously been reported that the  $\alpha$ -cleavage is independent of the precise sequence in the  $\alpha$ -cleavage site (Oliveira-Martins *et al.*, 2010; Tveit *et al.*, 2005). Differences in the protease activity could be one reason for the diverging observations of the proteolytic processing between cell lines, alternatively, the  $\alpha$ -PrPase may be constituted of different proteases that are able to possess  $\alpha$ -cleavage activity.

The cellular site at which the  $\alpha$ -cleavage takes place has been discussed and late compartments of the secretory pathway, endosomal/lysosomal

compartments and in lipid rafts have been suggested (Walmsley *et al.*, 2009; Tveit *et al.*, 2005; Taraboulos *et al.*, 1995). Controversy regarding the importance of the GPI anchor for the  $\alpha$ -cleavage exists and it has both been shown that the GPI-anchor is not a prerequisite for the  $\alpha$ -cleavage (Walmsley *et al.*, 2009; Tveit *et al.*, 2005) but that the  $\alpha$ -cleavage is dependent on being anchored to a membrane (Oliveira-Martins *et al.*, 2010). Furthermore, PrP<sup>C</sup> can be cleaved N-terminal to the  $\alpha$ -cleavage site generating a C2 fragment. This cleavage has been shown to take place at the cell surface (Watt *et al.*, 2005). In the expression system used in paper I, the N1-fragment could not be detected in the cell lysate, which suggests that the  $\alpha$ -cleavage took place at the cell surface releasing the N1 fragment directly from the cells into the extracellular medium.

In paper I, shedding of the majority of PrP<sup>C</sup> to the medium was due to a cleavage at the very C-terminal end, only a few amino acids from the GPI-anchor. The cleavage resulted in shedding of FL-S and C1-S. A pulse chase experiment was done in order to see if the deletion of the three aa in the  $\alpha$ -cleavage site also would interfere with the proteolytic cleavage in the extreme C-terminal end. The experiment showed that shedding of the PrP <sup>$\Delta$ 121-123</sup> was not affected by the deletion in the  $\alpha$ -cleavage site.

A minor fraction of released PrP<sup>C</sup> in the cell medium was migrating as GPI-anchored proteins as seen in gel electrophoresis. This minor fraction was released in association with exosomes, as isolated by differential ultracentrifugation. Exosomes can be purified using filtration and ultracentrifugation techniques and given their small size (around 50-100 nm), exosomes can be visualized only by electron microscopy after they have been purified from cell culture media or body fluids (Raposo *et al.*, 1996). A large number of proteins and lipids are enriched in exosomes and the most common exosomal proteins are used to characterize the exosomes after purification. In paper I, exosomes recovered by ultracentrifugation were characterized by western immunoblotting together with the exosome specific marker Tsg101. The morphology of the exosomes was determined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM technique uses backscattered (or reflected) electrons whereas in TEM the electrons are transmitted through the sample. In the samples from cell culture medium, vesicles with the size and morphology of exosomes were recovered. When staining the samples with anti-PrP immunogold the PrP were shown to be associated with exosomes.

The proteases involved in the cleavage events of PrP<sup>C</sup> have been controversially discussed. Several enzymes in the ADAM family have been suggested and also members of calcium-dependent calpain proteases (Hachiya *et al.*, 2011; Vincent *et al.*, 2001; Jimenez-Huete *et al.*, 1998). In paper I, the  $\alpha$ -cleavage was not affected when metalloprotease inhibitors were used, which demonstrated that metalloproteases were not responsible for the  $\alpha$ -cleavage as previously suggested. This is in line with newly presented results (Altmeppen *et al.*, 2011; Endres *et al.*, 2009). Instead, the metalloprotease inhibitors interfered with the protease-mediated shedding, showing that metalloproteases are involved in the shedding event. Interestingly, the amount of GPI-anchored PrP was increased in the cell medium from cells treated with metalloprotease inhibitors. This increase was probably due to a change in the route of PrP associated with exosomes or an effect of more PrP being present in the membrane and thus accessible for inclusion in exosomes.

Taken together, the results in paper I show that PrP<sup>C</sup> is released from the cell by three different mechanisms. The first mechanism releases an N-terminal fragment (N1) via the  $\alpha$ -cleavage, a second by proteolytic cleavage in the extreme C-terminal end generating GPI-anchorless FL-S and C1-S fragments, and a slower third process releasing a GPI-anchored PrP<sup>C</sup> in an exosomal fraction. It was also shown that a deletion in the  $\alpha$ -cleavage site inhibits the  $\alpha$ -cleavage and also that the  $\alpha$ -cleavage likely takes place at the cell surface. Finally it was shown that metalloproteases were not involved in the  $\alpha$ -cleavage of PrP<sup>C</sup> but instead responsible for the protease-mediated shedding and that PrP<sup>C</sup> could be shed in association with exosomes. These results provide important information and add further knowledge to the functional aspects of PrP<sup>C</sup> and possible roles in the pathogenesis of prion diseases.

#### 2.2.2 Paper II: Characterization of proteinase K-resistant N- and C-terminally truncated PrP in Nor98 atypical scrapie.

Classical scrapie has been recognized in sheep populations for more than 200 years and it has been shown that the disease has a clear link between susceptibility and genotype (Dawson *et al.*, 1998). However, in 1998, a newly identified form of scrapie was reported in Norway and it was subsequently named Nor98 (Benestad *et al.*, 2003). The new disease was distinct from classical scrapie with most cases appearing singly in flocks and affecting animals with genotypes considered to be highly resistant to classical scrapie.

In paper II, the proteinase K (PK) resistant PrP fragments from two Swedish cases of Nor98 atypical scrapie were characterized with regard to their molecular features. The fragmental pattern was analyzed by immunoblot mapping using a panel of antibodies to PrP directed to different epitopes spanning the PrP. The glycoprotein profiles of classical scrapie and Nor98 displayed a clear difference in their banding patterns after PK treatment. Classical scrapie has a characteristic three banding pattern with di-, mono-, and unglycosylated PrP bands. The most notable difference in the Nor98 samples, compared with the banding pattern of scrapie, was a prominent fast migrating band determined to be 7 kDa and was therefore designated Nor98-PrP7. This band has been reported to be 11-12 kDa (Arsac *et al.*, 2007; Nentwig *et al.*, 2007; Gretzschel *et al.*, 2006; Benestad *et al.*, 2003) or 8 kDa (Nentwig *et al.*, 2007). The disagreement about the size of this low molecular band is most likely due to different electrophoretic conditions or due to different PK conditions. Recently, the low molecular fragment was suggested to consist of two separate PK resistant fragments (Götte *et al.*, 2011). In paper II, the antigenic composition of Nor98-PrP7 revealed that this fragment comprised a midregion of PrP from around aa residue 85 to 148, corresponding to about 7 kDa. Furthermore, the Nor98-PrP7 band reacted with mAb L42 but not with mAb 6H4, which is reported to recognize an epitope partially overlapping the epitope of mAb L42. Indeed, mAb 6H4 appear to be more dependent of a conformational epitope of PrP than a linear epitope (unpublished data). This was evident when analyzing PrP fragments without a GPI-anchor. In these unpublished experiments, mAb L42 reacted with PrP fragments without a GPI-anchor, in contrast to mAb 6H4 that only reacted with PrP fragments containing an intact GPI-anchor. These findings are consistent with that the Nor98-PrP7 fragment is a result of PK truncation in both the N- and C-terminal parts of PrP. The truncation will result in that the Nor98-PrP7 lacks the GPI-anchor and could be the reason for why mAb 6H4 was not recognizing this band. In addition, deglycosylation did not change the distinct electrophoretic profile of Nor98-PrP7, which further prove that the small fragment corresponds to a central region of PrP that does not contain the glycosylation sites. N- and C-terminally truncated fragments spanning the midregion of PrP have only been observed in the genetic prion disorder Gerstmann-Sträussler-Scheinker disease. In addition, the small fragment in GSS and the Nor98-PrP7 fragment cover a region that can form amyloid fibrils partially resistant to PK digestion (Salmona *et al.*, 2003; Tagliavini *et al.*, 2001). However, no mutations were found in the Nor98-affected sheep that could be associated with a genetic explanation for the disease.

A previously unidentified PK-resistant C-terminal PrP fragment of around 24 kDa was detected and its PK-resistance was investigated. After deglycosylation this fragment migrated as a 14 kDa polypeptide and was designated PrP-CTF14. Its size and its interaction with C-terminal antibodies towards the C-terminal part of PrP and also its sensitivity to deglycosylation suggested that this fragment extended to the GPI-anchor. Interestingly, this band was in addition to mAb L42, recognized by mAb 6H4. This is in line with our new findings that mAb 6H4 recognize a conformational epitope. In addition, the existence of two PK-resistant PrP fragments, Nor98-PrP7 and PrP-CTF14, that share an overlapping region suggest that at least two distinct PrP conformations with different PK-resistant cores are present in brain extracts from Nor98 affected sheep.

In addition to these two bands, PK resistant bands migrating to masses of 33, 28 and 15 kDa were detected with antisera towards the mid-region of PrP. Neither the 15 kDa nor the 28 kDa fragment shifted in electrophoretic mobility after deglycosylation suggesting that these two fragments are C-terminally truncated. Despite the size of these fragments, the PK resistant material failed to react with antibodies recognizing epitopes suggested to be present on these fragments. In view of the findings for 6H4, these antibodies might also recognize conformational epitopes instead of linear. Also, it has been shown that PrP can maintain its tertiary structure although the sample had been boiled and treated with denaturants (Yuan *et al.*, 2005). It has also been shown that the core structure of amyloid fibrils can be packed so closely that even water molecules are excluded (Nelson *et al.*, 2005). It is therefore possible that certain epitopes could be inaccessible in the PK resistant fragments of Nor98.

In conclusion, the existence of two PK resistant fragments that share an overlapping region suggests that at least two distinct PrP conformations are present in the brain extracts from Nor98-affected sheep. Also, when analyzing the PK resistance, Nor98 PrP showed a reduced resistance compared to classical scrapie. The different banding pattern and PK resistance suggests different conformations of the classical scrapie and Nor98 PrP. The findings in paper II, together with observations of a distinct epidemiology and the lack of association with genetic changes suggests that Nor98 could be the result of an age-related spontaneous prion disease.

### 2.2.3 Paper III: Polymorphisms and variants in the prion protein sequence of European moose (*Alces alces*), reindeer (*Rangifer tarandus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*) in Scandinavia.

Chronic wasting disease (CWD) is an emerging prion disease of mule deer, white-tailed deer, elk, and moose. The efficiency by which CWD is spread suggests that transmission occurs primarily by horizontal route. Previous studies have revealed an association between polymorphisms in the prion protein sequence and susceptibility to CWD. Presently, the disease occurs only in parts of USA and Canada but has been found in South Korea via import from Canada (Kim *et al.*, 2005). During the 1980s a complex wasting syndrome in Swedish moose, Moose Wasting Syndrome (MWS), was described. The diseased animals showed signs of central nervous disturbances, lesions in mucosal membranes and intestines and atrophied lymphoid organs. Unusual behaviors like circling, no fear of man and anorexia were displayed. However, contemporary pathological investigations indicated no association with a spongiform encephalopathy.

In paper III the genetic diversity of the ORF in the *PRNP* and the aa sequence of Scandinavian cervids were analyzed and compared with variations described in the North American cervid population. A unique variant in the European moose PrP codon 109 was found and both homozygous (K/K or Q/Q) and heterozygous (K/Q) genotypes were shown. In contrast, Alaskan moose and other cervids sequenced in paper III were homozygous for 109K/K. The 109 codon is situated in a positively charged cluster, which is highly conserved between species and only four aa N-terminal to the  $\alpha$ -cleavage site. Human PrP sequence variants in this cluster are associated with a GSS phenotype (Hsiao *et al.*, 1989) and transgenic mice carrying mutations in the region developed neurodegenerative diseases spontaneously (Hegde *et al.*, 1999).

In order to elucidate if there was any link between the K109Q variant and the MWS animals, a single-nucleotide polymorphism (SNP) analysis was performed. Samples collected during the outbreak of MWS were used together with time matched healthy animals. The observed genotype proportion of the heterozygous K/Q was higher among the MWS animals compared to the healthy (0.46 and 0.35 respectively). When comparing the proportion of the genotypes A/C to C/C, a significantly greater proportion of A/C was found in the MWS animals than in healthy animals, 0.93 and 0.71, respectively. These



data could suggest a possible association between MWS and the K109Q polymorphism.

In reindeer, codon 225 varied with either heterozygous SY or homozygous YY or SS animals. In comparison, the codon 225 in mule deer is polymorphic but with 225S and 225F where the heterozygous 225SF variant were linked to reduced susceptibility. All species in paper III were homozygous for Met at position 132. This position corresponds to codon 129 in humans and in Rocky Mountain elk the 132MM individuals were over-represented among CWD-positive animals (O'Rourke *et al.*, 1999).

There is currently no evidence that CWD exist in cervids in Scandinavia. Approximately 13,000 brain stem samples have been collected from cervids of different species in the EU and Norway and no TSE positive results have been found (EFSA, 2010). Despite this, examining the *PRNP* genetic diversity is of great interest as an introduction of CWD among wild species is possible. It has been shown that distinct CWD strains exist (Angers *et al.*, 2010) and interspecies transmission can alter CWD host range and the potential of interspecies transmission of CWD will increase as the disease spreads. Also, as the TSE agents have the potential to cross the species barrier it is a possibility that cervids in Scandinavia could be exposed to scrapie prions. To date, most studies and experimental work have suggested that the potential for CWD transmission to humans is low. A still ongoing multi-year study in non-human primates reported results that suggest that human may be resistant to some strains of CWD (Race *et al.*, 2009). Further, in a recent study, a CWD isolate from white-tailed deer was inoculated into Tg mice expressing human PrP but no signs of disease were observed in the mice (Wilson *et al.*, 2012).

Taken together, the PrP sequence of European moose, reindeer, roe deer and fallow deer in Scandinavia has high homology to the PrP sequence of North American cervids. This study also confirmed that the Scandinavian cervids carries polymorphisms that are compatible with a susceptibility to CWD. A unique aa variant was found at position 109 in the PrP of European moose. Also, a difference in the observed genotype proportions of heterozygous and homozygous animals at codon 109 were found in the MWS animals compared to healthy animals.



### 3 Conclusions and future perspectives

Defining the mechanisms behind the formation of PrP<sup>Sc</sup> from PrP<sup>C</sup> has become one of the central issues in understanding the pathogenesis of prion diseases. It is likely that the conformational diversity found in PrP<sup>Sc</sup> reflects a possible conformational diversity necessary for the function of PrP<sup>C</sup>. In relation to this, establishing the physiological role of PrP<sup>C</sup> is highly important as the protein may fail to carry out its normal function when converted to the diseased isoform. And, as stated by the nobel laureate Kurt Wütrich in Munich 2003, “PrP<sup>C</sup> is the key”, in response to the numerous experiments done exclusively on the disease-associated PrP<sup>Sc</sup>. Knowledge about the cleavages that occur in PrP<sup>C</sup> is an important section in this part since these cleavages are likely to play an important role both in normal function for PrP<sup>C</sup> but also for the conversion into PrP<sup>Sc</sup> and pathogenesis of prion diseases. Blocking the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> would be a good practical therapeutic approach for preventing prion diseases. One hypothesis is for example that the  $\alpha$ -cleavage disrupts the region necessary for the conformational change and thereby prevents the formation of PrP<sup>Sc</sup>. Defining the proteases involved in the cleavages could provide novel approaches for therapeutic interventions against prion diseases.

In this thesis, the proteolytic cleavages and shedding of the cellular prion protein are investigated in order to gain further insight into the function and intercellular transmission of PrP<sup>C</sup>. Here, it is shown that PrP<sup>C</sup> can be shed into the extracellular medium by three different mechanisms. In future work, these three mechanisms are important to observe when analyzing the different cleavages of PrP<sup>C</sup> and also for getting the complete view of the processing when performing experiments with PrP<sup>C</sup>. In this thesis, the proteases involved in the different cleavages were studied and different inhibitors were used. These inhibitors only partly interfered with proteases involved in the shedding of the PrP<sup>C</sup>. This probably reflects the very complex systems involved in the

cellular processing of PrP<sup>C</sup>. Earlier findings also point to the importance of knowledge about the cell culture model used in studies of PrP as different cell lines can possess differences in their specific protease activities (Zhao *et al.*, 2006). The use of *in vitro* models is a powerful tool but it is also reasonable to be critical against inconsistent or contradictory results. However, this does not mean that the results are wrong or not useful and careful considerations of these inconsistencies could instead give new insights. An example of this is the set of experiments performed in this thesis to investigate the role of exosomes in PrP<sup>C</sup> processing. Overexpression of PrP might for example lead to release of PrP in association with exosomes as a consequence of removal of unwanted molecules via exosome shedding, which is one of their suggested functions. Further investigations into the roles of PrP in exosomes need to be done. So far, exosomes have been shown to contain an ever increasing number of proteins and the characteristics of exosomes are still not completely reliable. A protein that is only present in exosomes would be a good marker for characterization of exosomes together with new techniques to visualize exosomes. Cryo-TEM is a form of TEM where the sample is studied at cryogenic temperatures and the structure of the sample remains native, as no dehydration is needed. This technique could be used more in the future when working with exosomes. Interesting future investigations would also be for example to include additional protease inhibitors, not only for metalloproteases but also for calpain and other proteolytic enzymes, to finally determine which protease(s) that are responsible for the different cleavages. In regard to this, an experimental setup could be used in which the aa sequence of interest, for example the aa region around the  $\alpha$ -cleavage site, is inserted between two molecules used for detection. This construct is then recombinantly expressed and the model substrate containing the  $\alpha$ -cleavage site will then be subjected to different cleavage enzymes.

During the last decade, active surveillance programs for TSEs in small ruminants have been performed in Europe. In many countries, this has led to the detection of cases of Nor98 atypical scrapie. In the active surveillance for TSEs in sheep in Sweden a number of Nor98 atypical scrapie have been found. The influence of PrP polymorphisms on the susceptibility to scrapie has earlier been investigated and certain genotypes, such as VRQ/VRQ are known to be highly susceptible while the ARR/ARR are considered resistant. One concern in relation to this has been that Nor98 affected sheep have had genotypes, which are considered to be resistant to scrapie infection. This has turned out to be problematic, as many countries have adopted control programs that promote breeding for the Scrapie-resistant genotype. The origin of Nor98 is unknown

but it is suggested that it may represent a spontaneous TSE of sheep, as affected Nor98 sheep generally are older than scrapie infected sheep and only single Nor98 cases in a flock are detected. This together with other knowledge has led to the view that Nor98 is not transmissible to humans and animals. However, it was recently shown that samples containing Nor98 infectivity could be PrP<sup>Sc</sup>-negative (Andreoletti *et al.*, 2011). These results indicate that the exposure risk to Nor98 may be higher than commonly believed and also that the prevalence of Nor98 is underestimated in the affected flocks. Also, in a study made by Le Dur *et al.* (2005) it was reported that Nor98 efficiently could be transmitted to Tg mice expressing ovine PrP. Finally, the risk of Nor98 to cross species barrier that naturally limits the transmission risk is insufficiently investigated and underline further investigations. And, in regard to control programs, it is important to see if the disease occurs sporadically or if it can be transmissible.

The origin and mode of transmission of CWD is unknown but based on epidemiological data, the transmission is thought to occur horizontal. Research has recently shown that the disease can be transmitted by contaminated soil and also that infected deer's saliva can contain infectivity (Mathiason *et al.*, 2006; Miller *et al.*, 2004). The European food Safety Authority presented a survey aimed at detecting the possible presence of CWD in wild and farmed cervids in the EU and Norway. In line with this, a recommendation was made to investigate the *PRNP* genetic diversity of European cervids and to compare it with variations described in the North American cervid population. Here, it was shown that PrP genotypes exist in cervids in Scandinavia that are similar to the PrP genotypes of North American cervids. This confirms that cervids in Scandinavia have a *PRNP* genetic background that is compatible with CWD. In the 1980s, a wasting syndrome in Swedish moose (MWS) was described. Today, there is still no definite answer to the underlying cause of that syndrome. Pathological investigations indicated no associations with a TSE disease at that time. Here, we show that a K109Q polymorphism in European moose could be associated with MWS. However, further studies need to be done and it would be interesting to carry out more genetic analyses on the historical MWS samples. It would also be interesting to analyze brain material collected during the time of outbreak to see if today's methods for diagnosis can demonstrate a connection to a TSE disease.

The interaction of PrP<sup>C</sup> with A $\beta$ -peptides from the amyloid precursor protein (APP) has been demonstrated in several studies (Chen *et al.*, 2010; Lauren *et al.*, 2009). Despite this, the functional relevance of an interaction

between them is still unclear. Both PrP and APP are membrane proteins that are subjected to complex proteolytic processing and they are also released from cells by similar proteolytic activities. Both have been shown to be associated with exosomes and pre-fibrillar intermediates mediate toxicity in both Alzheimer's disease (AD) and prion diseases. It would be interesting to use our expression system and co-express the proteins and then analyze the different cleavage patterns and shedding products as well as the association with exosomes for both PrP and APP.

In summary, the proteolytically cleavages and shedding of the PrP<sup>C</sup> have been investigated in this thesis. Also, the molecular properties of Nor98 and the genetic diversity within cervids in Scandinavia have been examined. Throughout the study, it has been evident that it is crucial to evaluate both the methods and analysis used in the different projects. In addition to this, the antibodies used for detection of PrP are important to critically evaluate since antibodies claimed to recognize the same epitope not necessarily are functional in certain conditions.

Although several issues remain in the field of prion research, the present results will be one part in the continued hard work of solving these questions. Hopefully one day the mystery of PrP, its normal cellular function and its role in disease will be solved. But for now, there are still plenty of questions to answer.

## 4 Populärvetenskaplig sammanfattning

Prionsjukdomar eller transmissibla spongiforma encefalopatier är en grupp dödliga och överförbara sjukdomar som kan drabba både människor och djur. Sjukdomarna orsakas av ett speciellt smittämne som kallas prioner. Själva ordet prion är en förkortning av proteinlik infektiös partikel och detta smittämne skiljer sig från andra smittämnen eftersom ingen nukleinsyra påvisats i samband med sjukdomen. Individer som insjuknar får en demensliknande sjukdomsbild med symtom som koordinations- och beteendestörningar, minnesförlust och depression. Sjukdomarna ger inte upphov till någon reaktion från kroppens immunförsvar eftersom prionproteiner finns naturligt i framförallt nervceller hos alla däggdjur. Det cellulära, normala prionproteinet har fått beteckningen PrP<sup>C</sup> där C står för cellulär. Funktionen hos PrP<sup>C</sup> är ännu okänd, men allt talar för att det har en viktig biologisk funktion eftersom genen för prionproteinet är väldigt välkonserverad mellan arter. Några exempel på föreslagna funktioner är igenkänning, signalering, kopparbindning med flera.

Genom en ännu okänd mekanism så kan PrP<sup>C</sup> genomgå en förändring och bilda det sjukliga prionproteinet som betecknas PrP<sup>Sc</sup> där Sc står för scrapie. Det är alltså samma protein men i en helt ny form och med helt nya egenskaper. Proteinet blir väldigt motståndskraftigt mot nedbrytning och PrP<sup>Sc</sup> ansamlas i cellerna. PrP<sup>Sc</sup> kan även fungera som mall och göra så att normala prionproteiner får en felaktig konformation. De nya egenskaperna är bland annat att prionerna är extremt motståndskraftiga mot proteaser, UV-ljus, kemikalier, värme m.m. Man har visat att en syntes av det normala, cellulära prionproteinet är nödvändigt för sjukdom.

För att kunna förhindra prionsjukdomar är det viktigt att förstå proteinets normala funktion. I avhandlingen har bland annat olika klyvningar av prionproteinet analyserats. Studierna har även påvisat att prionproteinet kan utsöndras samtidigt från cellen via tre olika mekanismer. Två av mekanismerna utgörs av två olika klyvningar medan den tredje mekanismen involverar exosomer. Exosomer är väldigt små cell-liknande blåsor som skulle kunna vara en viktig partikel för prionproteinets normala funktion, men även viktig i fråga om smittspridning.

Nor98 är en prionsjukdom hos får som identifierades för första gången i Norge 1998, därav namnet. Sverige har haft ett flertal fall av Nor98 de senaste åren. Till skillnad från scrapie som är en smittsam prionsjukdom hos får, har man hittills inte hittat några bevis för att Nor98 smittar mellan djur. Eftersom ursprunget till sjukdomen fortfarande är okänd är det viktigt att försöka ta reda på mer om denna sjukdom. I avhandlingen har de genetiska och molekylära egenskaperna hos Nor98 analyserats och jämförts med scrapie. I studien framkom att Nor98 antagligen representerar en spontan prionsjukdom. Det påvisades även att Nor98 förmodligen inte har någon genetisk bakgrund som ger upphov till sjukdomen och att den molekylärt skiljer sig från scrapie.

Chronic wasting disease (CWD) är en smittsam prionsjukdom som drabbar hjorddjur i Nordamerika. I en av studierna som presenteras i denna avhandling har vi analyserat den gen som kodar för prionproteinet hos skandinaviska hjorddjur. I studierna framkom att denna gen är väldigt lik den som kodar för prionproteinet hos Nordamerikanska hjorddjur och som kan sammankopplas med en risk att smittas av CWD. Hos älgar hittade vi dessutom en helt unik variant i sekvensen som kodar för prionproteinet. Denna variant skulle eventuellt kunna kopplas till en älgsjukdom som fanns i Sverige under mitten av 1980-talet och i början av 1990-talet.

Idag finns varken läkemedel eller vaccin tillgängligt för att bota eller hindra prionsjukdomar. Resultaten som framkommit i denna avhandling är viktiga eftersom de kan vara intressanta ledtrådar till prionproteinets funktion och också för förståelse av smittspridning och omvandling till den sjukliga varianten av prionproteinet.



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