The Prion Protein in Normal Cells and Disease

Studies on the Cellular Processing of Bovine PrP^C and Molecular Characterization of the Nor98 Prion

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Cover illustration: (Left) Western immunoblot of time-course on the PK-resistance of Nor98 (left) and scrapie (right) PrP^{Sc}. (Right) SEM image on exosomes from boPrP-expressing cells on a copper support.
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Abstract

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Transmissible spongiform encephalopathies (TSEs) are also known as prion diseases. The unusual infectious agent is composed largely, if not entirely, of a proteinase resistant aberrantly folded isoform of the prion protein (PrP^{Sc}). PrP^{Sc}, through an unknown mechanism, is believed to impose its aberrant conformation onto the host-encoded cellular prion protein, PrP^C. The infectious particle is designated a "prion", an acronym for *proteinaceous infectious particle*, to distinguish it from conventional pathogens such as viruses and bacteria.

The essential role of PrP^C in the pathogenesis of prion diseases motivated the detailed study of the cellular processing, turnover and release of bovine PrP^C ($boPrP^C$) performed in this thesis. $BoPrP^C$ was found to be subjected to two distinct proteolytic cleavage events, generating an N-terminal and a C-terminal $boPrP^C$ fragment. Both PrP fragments were released from the cell. Moreover, in normal bovine brain a C-terminal fragment was found, suggesting that similar proteolytic processing events occur *in vivo*.

The finding that $boPrP^C$ in addition to a protease-mediated release also was released in association with exosomes, provide important information in relation to functional aspects of PrP^C and possible roles of exosome-associated PrP in pathogenesis of prion diseases. Taken together, these results indicate that release of $boPrP^C$ from cells represent an important step in the normal cellular processing of $boPrP^C$.

Nor98 is a recently recognised prion disease in sheep. The molecular characterization of the Nor98 prion showed that the unique PK-resistant PrP fragments present in Nor98-affected sheep display striking similarities to those reported from individuals affected by the human prion disorder Gerstmann-Sträussler-Scheinker syndrome (GSS). Interestingly, GSS is always associated with amino acid substitutions in the PrP. Differently, no disease-causing changes in the PrP of Nor98-affected sheep have been found in the affected sheep in Sweden. These findings together with observations of a distinct epidemiology, suggest that Nor98 could be the result of a spontaneous conversion of PrP^C into PrP^{Sc}, similar to that proposed for sporadic Creutzfeldt-Jakob disease and sporadic fatal insomnia.

Keywords: prion, Nor98, scrapie, BSE, exosomes, shedding, C1, TSE

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To my mum and dad

"Few things are impossible to diligence and skill. Great works are performed not by strength, but perseverance."

Samuel Johnson
English author, critic, & lexicographer (1709 - 1784)

Contents

Appendix

Papers I-III

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Zhao, H.*, Klingeborn, M.*, Simonsson, M. and Linné, T. (2006) Proteolytic cleavage and shedding of the bovine prion protein in two cell culture systems. *Virus Res.* 115: 43-55.
(*Equal contribution)

II. Klingeborn, M., Wik, L., Johansson, H. and Linné, T. (2006) Exosome- and Protease-mediated Shedding of the Bovine Prion Protein is Unaffected by Deletion of the C1 Cleavage Site. *Manuscript.*

III. Klingeborn, M., Wik, L., Simonsson, M., Renström, L.H.M. 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.

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Abbreviations

aa	amino acid
boPrP	bovine PrP
BSE	Bovine Spongiform Encephalopathy (mad cow disease)
CJD	Creutzfeldt-Jakob disease
huPrP	human PrP
moPrP	mouse PrP
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
ORF	open reading frame
ovPrP	ovine PrP
PIPLC	phosphatidylinositol-specific phospholipase C
PK	proteinase K
PNGase F	peptide: N-glycosidase F
PRNP/Prnp	PrP gene
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{Nor98}	PK-resistant Nor98 disease-associated isoform of PrP
PrP-res	PK-resistant PrP
PrP^{Sc}	PK-resistant disease-associated isoform of PrP
PrP*	the infectious entity in prions, a hypothetical abnormal isoform of PrP, different from both PrP^{C} and PrP^{Sc}
PrP 27-30	PK-resistant core of PrP^{Sc} resulting from exogenous PK-treatment
rPrP	recombinantly expressed PrP
SHaPrP	Syrian hamster PrP
TSE	Transmissible Spongiform Encephalopathy

Background

One of the more significant paradigm shifts in molecular medicine during the last 25 years was the move away from the clinical-neuropathologic classification of scrapie and Creutzfeldt-Jakob disease (CJD) as ‘‘transmissible spongiform encephalopathies’’ (TSEs) to the recognition of the central causal and pathogenic roles played by the prion protein (PrP) and classification of these and related neurodegenerative disorders as “prion diseases” (Prusiner *et al.*, 1998). The animal syndromes include scrapie of sheep, bovine spongiform encephalopathy (BSE), chronic wasting disease of mule deer and elk (CWD), transmissible mink encephalopathy, and feline spongiform encephalopathy. Recently also atypical variants of prion diseases in animals such as Nor98 in sheep and BASE in cattle, have been discovered, possibly representing a group of sporadic prion diseases in animals (Benestad *et al.*, 2003; Casalone *et al.*, 2004; Watts, Balachandran & Westaway, 2006). The list of human prion diseases includes sporadic, familial, iatrogenic, and variant CJD (sCJD, fCJD, iCJD, and vCJD, respectively); Gerstmann-Sträussler-Scheinker syndrome (GSS); sporadic and familial fatal insomnia (sFI and FFI, respectively); and kuru. In the past, these diseases were thought to be caused by ‘‘slow viruses’’ because the ‘‘infectious’’ agents that transmit these diseases are possible to filter and incubation times are typically months to years. Several unconventional properties regarding the transmissible agent, such as resistance to high temperature (Brown *et al.*, 2000b; Gordon, 1946), high pressures (Brown *et al.*, 2003), formaldehyde treatment (Gordon, 1946), and UV-radiation (Alper *et al.*, 1967; Alper, Haig & Clarke, 1966; Gibbs, Gajdusek & Latarjet, 1978; Latarjet *et al.*, 1970) added to the obscurity of these diseases (Prusiner, 1982; Prusiner, 1998). The etiology remained perplexing, because approximately 99% of human prion diseases cannot be connected to infection and the histological features are dissimilar to those of a typical infectious process. An additionally puzzling characteristic is that 10% to 15% of human prion diseases are dominantly inherited, including all cases of GSS, all cases of FFI, and approximately 10% of CJD cases (fCJD). There was no precedent to explain how a disease could be both infectious and genetic.

The molecular mechanisms fundamental to such contradictory associations in prion diseases have been revealed over the past two decades, beginning with the discovery that the ‘‘infectious’’ agent that transmits scrapie is composed largely, if not entirely, of a protease-resistant, 27-kDa to 30-kDa protein (Prusiner, 1982). The infectious particle was designated a ‘‘prion’’, an acronym for *proteinaceous infectious particle*, to distinguish it from conventional pathogens such as viruses and bacteria. The polypeptide constituting the proteinase K (PK)-treated prion particle was designated PrP 27-30. This was followed by the discovery that PrP is encoded by a single copy mammalian gene, designated *PRNP* in humans, sheep, and cattle and *Prnp* in mice and hamsters (Oesch *et al.*, 1985). In humans, the *PRNP* gene is located on chromosome 20, in mice on chromosome 2 (Sparkes *et al.*, 1986) and, in cattle and sheep on chromosome 13 (Iannuzzi *et al.*, 1998). PrP 27-30 was found to be derived from a larger 30-kDa to 35-kDa protein, designated $PrP^{Sc(rapie)}$, in scrapie-infected animals, and PrP^{Sc} was found to be derived from a

normal constitutively expressed protease-sensitive PrP isoform, designated PrP^{C} (Meyer *et al.*, 1986). Subsequently, all of the dominantly inherited forms of prion disease were genetically linked to mutations of, or insertions in, the *PRNP* gene (DeArmond & Prusiner, 1996; Gambetti & Parchi, 1999; Hsiao *et al.*, 1989).

Introduction

How can a protein have the characteristics of a conventional contagious agent? Evidence from many laboratories indicates that the mature, full-length PrP molecule can exist in at least two conformations without additional chemical modifications: a normal, nonpathogenic isoform that is largely α-helical with little β-sheet content, characteristic of the constitutively expressed normal cellular conformer (PrP^{C}); and an abnormal, pathogenic isoform with less α-helical and more β-sheet content, characteristic of the disease-associated isoform (PrP^{Sc}). Furthermore, PrP^{Sc} can interact with the PrP^{C} expressed by mammalian cells and cause the PrP^{C} to take on an identical β-sheet conformation, which starts off a self-perpetuating process that results in increasing PrP^{Sc} concentrations and prion infectivity titres (Prusiner*, et al.*, 1998). That a protein can have the ability to adopt two conformations without chemical modification is not a feature restricted to PrP. For example, several other proteins involved in the pathogenesis of diseases called "protein misfolding disorders" (PMDs) have also been shown to display this property under certain circumstances, for a review see (Soto, Estrada & Castilla, 2006). Furthermore, the prion concept has been extended to explain three unusual genetic elements of the yeast *Saccharomyces cerevisiae* and one of the filamentous fungus *Podospora anserina*. The three yeast proteins - the nitrogen regulatory protein Ure2p, the ribosome termination factor Sup35, a nonessential protein of unknown function denoted Rnq1, and the Het-s protein in *P. anserina* - have prion-like characteristics: like prions, they can undergo conformational changes, acquire β-sheet structure, and selfaggregate into intracellular, β-rich, protease-resistant agglomerate (Uptain & Lindquist, 2002).

From studies of mammalian and yeast prions four new concepts have crystallised (Prusiner, 2001; Weissmann, 2004). First, the only known pathogens that are devoid of nucleic acids are prions. All other infectious agents possess genomes that direct the synthesis of their progeny. Second, like other neurodegenerative disorders, prion diseases can manifest as either sporadic or genetic diseases. Unlike other neurodegenerative disorders, however, prion diseases can be infectious – infectious prions are formed irrespective of the initiating molecular event. Third, neurodegeneration in prion diseases results from the accumulation of PrP^{Sc} or some abnormal conformer of PrP^{C}, and PrP^{Sc} is almost invariably detected in prion-infected tissues and cells, which because of its high β-sheet content, is relatively resistant to degradation by cells, including neurons (DeArmond & Bouzamondo, 2002). Fourth, PrP^{Sc} can take on a range of pathogenic conformations that give prions the strain features associated with different disease phenotypes (Telling *et al.*, 1996).

The protein-only hypothesis

The extraordinary resistance of prions to radiation (Alper*, et al.*, 1967; Alper, Haig & Clarke, 1966) led early on to the suggestion that they might be lacking nucleic acid and consist only of protein (Griffith, 1967). The "protein-only" hypothesis as stated by Stanley Prusiner proposes that the prion is a proteinaceous infectious

particle that lacks nucleic acid and that the infectious, abnormal conformer of PrP^{C} is propagated autocatalytically (Prusiner, 1982; Prusiner, 1997; Prusiner, 1998). Models for the specific mechanism of prion propagation is discussed in detail below (*Propagation of PrP^{Sc}*). It is, however, not clear if the infectious entity is composed of PrP^{Sc} or PrP* (an abnormal conformer of PrP^{C} different from PrP^{Sc}). The presence of a small ligand in addition to PrP^{Sc}/PrP* in the prion particle, cannot be completely excluded.

There are a number of lines of evidence that supports the protein-only hypothesis (modified from (Prusiner, 1998; Soto, Estrada & Castilla, 2006)):

- PrP^{Sc} and scrapie infectivity co-purify when biochemical and immunologic procedures are used. That is, highly purified preparations of PrP^{Sc} transmit the disease (Prusiner *et al.*, 1984).
- Procedures that inactivate PrP^{Sc} markedly reduce or eliminate infectivity (Prusiner *et al.*, 1993).
- Levels of PrP^{Sc} are directly proportional to prion titers. Non-denatured PrP^{Sc} has not been separated from scrapie infectivity (Gabizon *et al.*, 1988).
- No evidence exists for a virus-like particle or a nucleic acid genome (Safar *et al.*, 2005). The infectious agent is not affected by procedures that normally destroy nucleic acids (Alper, *et al.*, 1967; Prusiner, 1982).
- Accumulation of PrP^{Sc} is almost invariably associated with the pathology of prion diseases, including PrP amyloid plaques that are pathognomonic.
- PrP gene mutations are genetically linked to inherited prion disease and cause formation of PrP^{Sc}.
- PrP gene knockout mice are not susceptible to prion infection. As the substrate necessary for PrP^{Sc} formation have been eliminated both prion disease and prion replication is prevented (Bueler *et al.*, 1993). In addition, overexpression of PrP^{C} increases the rate of PrP^{Sc} formation, which shortens the incubation time.
- Species variations in the PrP sequence are responsible, at least in part, for the species barrier that is found when prions are passaged from one host to another.
- Chimeric and partially deleted PrP genes change susceptibility to prions from different species and support production of artificial prions with novel properties that are not found in nature (Legname *et al.*, 2004; Legname *et al.*, 2005).
- Prion diversity is enciphered within the conformation of PrP^{Sc}. Strains can be generated by passage through hosts with different PrP genes.
- Accumulation of PrP^{Sc} in FFI distinct from that found in fCJD with the same D178N mutation, suggest distinct prion strains with diverse pathological potential (Montagna *et al.*, 2003).
- PrP^{Sc} can induce the conversion of PrP^{C} *in vitro* in an autocatalytic fashion (Caughey *et al.*, 1999; Kocisko *et al.*, 1994; Saborio, Permanne & Soto, 2001).

In addition, even though not yet achieved completely in mammalian systems, the protein-only hypothesis has been demonstrated to be valid with the use of yeast prions (see below under *Prion Strains* and (King & Diaz-Avalos, 2004; Sparrer *et al.*, 2000; Tanaka *et al.*, 2004; Tanaka *et al.*, 2006)), albeit not as much *bona fide* prion proteins as proteins with prion-like properties.

Even though many lines of evidence support the protein-only hypothesis, alternative models have been suggested for the composition of the TSE agent and the pathogenesis of TSEs (Chesebro, 1999; Kimberlin, 1982; Manuelidis, 2003; Weissmann, 1991). All of these alternative models predicates in one way or another that a TSE-specific nucleic acid is crucial for prion propagation and/or modulating strain-specific characteristics of the TSE agent. However, the failure to identify a TSE-associated nucleic acid in spite of intense efforts in many laboratories have led to that these models have lost much support (Safar*, et al.*, 2005; Weissmann, 2004).

Prion diseases

Some of the distinctive features of TSEs or prion diseases are neuronal vacuolation (spongiosis), neuronal death, and explicit glial reactions. In addition, a defining characteristic is the deposition, mainly in the brain and lymphoreticular tissues, of PrP^{Sc}. Also, no adaptive immune responses are elicited upon infection, most likely because the mammalian immune system is largely tolerant to PrP of the same species. This is not surprising, given that many cells in neural and extraneural compartments express PrP^{C}. Although TSEs are by definition transmissible, a growing number of *Prnp*-associated non-infectious neurodegenerative proteinopathies are now also being recognized (Chiesa *et al.*, 2003; Tateishi & Kitamoto, 1995; Weissmann & Flechsig, 2003).

Scrapie - the prototype prion disease

Scrapie, the prototypic prion disease affecting sheep and goats has been recognized in sheep and goat populations for more than two centuries making it the oldest described member of the TSEs (Stockman, 1913). An important breakthrough was the experimental transmission of scrapie to goats reported in 1939 (Cuille & Chelle, 1939). Scrapie is considered to occur worldwide with a small number of exceptions. Australia and New Zealand eradicated scrapie following its introduction in imported sheep and are now regarded as being free of scrapie. A prominent characteristic of scrapie is pruritus, hence the name of the disease. Sheep that are affected sometimes rub against solid objects to the point where wool is scraped off (Williams, 2003).

Extensive retrospective studies together with a review of world literature led to the conclusion, published in 1987, that scrapie had never passed to humans despite opportunities to do so over the 250 years during which the disease had contaminated sheepmeat entering the human food chain (Brown *et al.*, 1987). It was also recently reported that Gombojav and co-workers (Gombojav *et al.*, 2003) failed to infect Hu/Mo chimeric transgenic mice with either sheep or mouse-

adapted scrapie, further supporting the above view. Gajdusek and colleagues also reported difficulties infecting chimpanzees with sheep scrapie (Gibbs & Gajdusek, 1973).

Scrapie is a very rare disese in Sweden, in fact the only documented cases were diagnosed in 1986 in two ewes from the same flock (Elvander, Engvall & Klingeborn, 1988). The diagnosis was based on clinical history and patho-anatomical changes only and to verify the diagnosis, inoculations into C57/Bl mice and Swedish landrace goats were performed. Mice started exhibiting symptoms of scrapie 13 months post inoculation (p.i.) and histopathological examination revealed typical signs of scrapie. Goats started showing signs of scrapie 16 months p.i. Typical signs of scrapie, such as vacuolisation and spongiform degeneration, were seen also in goats upon histopathological examination. These inoculation experiments confirmed that these sheep were indeed affected by scrapie. Western immunoblotting performed recently on brain homogenates from one of these affected sheep also showed that the molecular characteristics displayed by the PrP^{Sc} are those of classical scrapie (Klingeborn *et al.*, 2006b).

Susceptibility alleles in scrapie

Common polymorphisms at codons 136 (V or A), 154 (H or R) and 171 (R or Q) of the ovPrP define alleles (represented by the single letter amino-acid code in the order 136:154:171) that are linked to survival time of sheep exposed to natural scrapie or experimental scrapie and BSE (Belt *et al.*, 1995; Bossers *et al.*, 1997; Bossers, de Vries & Smits, 2000; Bossers, Harders & Smits, 1999; Bossers *et al.*, 1996; Goldmann *et al.*, 1994; Hunter *et al.*, 1996). The relative resistance to classical scrapie infection in sheep carrying the five most common ovPrP alleles are ARR>AHQ>ARH>ARQ>VRQ.

Cell-free conversion of PrP^{C} offer an *in vitro* model, in which relative amounts of formed proteinase K (PK)-resistant PrP mirror important biological features of TSEs at the molecular level (Bossers*, et al.*, 1997; Bossers, de Vries & Smits, 2000; Bossers, Harders & Smits, 1999; Bossers *et al.*, 2003; Caughey *et al.*, 1995; Raymond *et al.*, 1997). This method has shown that V_{136}- and wt-PrP^{C} (i.e. A_{136}-PrP^{C}) are readily converted into PrP-res by a range of types of PrP^{Sc} isolated from sheep having different PrP genotypes. In contrast, R_{171}-PrP is hardly converted into PrP-res (Bossers*, et al.*, 1997; Bossers, de Vries & Smits, 2000; Bossers, Harders & Smits, 1999; Bossers*, et al.*, 2003; Raymond *et al.*, 2000). These observations demonstrate that the conversion efficiencies of distinct ovPrP^{C} allelic variants into a PK-resistant isoform can be directly correlated to the level of susceptibility to scrapie *in vivo*.

BSE and vCJD

The BSE epidemic in the UK has caused more than 180,000 cases since 1986 and the total number of infected animals have been estimated at around one million (Collinge, 2001). A range of exotic zoo animals of the felidae and bovidae families were also infected through the consumption of BSE-contaminated bovine meat products (Cunningham *et al.*, 2004; Sigurdson & Miller, 2003) and data strongly

support a link between BSE and variant Creutzfeldt-Jakob disease (vCJD) in humans (Bruce *et al.*, 1997; Collinge, 1999; Hill *et al.*, 1997; Scott *et al.*, 1999). Transmission of bovine prions across the bovine-human species barrier could be due to either a genetic disposition of the human prion protein (Tabrizi, Elliott & Weissmann, 2003) or an intrinsic property of the bovine prion protein and its metabolism (Collinge, 1999), or both.

In response to the discovery that the BSE prion probably had infected humans a committee was formed, The BSE Inquiry, which was given the task to establish and review the history of the emergence and identification of BSE and variant CJD in the United Kingdom, and of the action taken in response to it up to 20 March 1996 (http://www.bseinquiry.gov.uk/). Many important findings were disclosed in the BSE Inquiry report, and some of the most important were (Matravers, Bridgeman & Ferguson-Smith, 2000):

• The cases of BSE identified between 1986 and 1988 were not index cases, nor were they the result of the transmission of scrapie. They were the consequences of recycling of cattle infected with BSE itself. The BSE agent was spread in meat-and-bone meal (MBM).

• BSE probably originated from a novel source early in the 1970s, possibly a cow or other animal that developed disease as a consequence of a gene mutation or a spontaneous conformational change of PrP (Scott *et al.*, 2005). The origin of the disease will probably never be known with certainty.

• Rendering methods have never been capable of completely inactivating TSE agents. However, changes in rendering methods in the mid to late 1970's might possibly have resulted in a larger fraction of BSE prions to survive in the MBM fed to cattle, although experimental data is inconclusive in this regard.

The incidence of vCJD in the UK increased each year from 1996 to 2001 (www.dh.gov.uk and www.cjd.ed.ac.uk), eliciting fears of a large upcoming epidemic. Since then, however, the incidence of vCJD in the UK seems to be stabilizing and may in fact even be falling. Consequently, there is considerable hope that the total number of vCJD victims will be fairly small even though concerns over longer incubation times in individuals carrying certain polymorphisms in the PrP gene have been raised (Collinge *et al.*, 2006; Valleron *et al.*, 2006; Valleron *et al.*, 2001). A polymorphism at position 129 in huPrP have been shown to influence the incubation period and phenotype in iCJD and kuru (Brown *et al.*, 2000a; Collinge*, et al.*, 2006). All vCJD cases diagnosed to date have been homozygous for the M_{129} allele except one heterozygote infected via blood-borne transmission (Hewitt *et al.*, 2006). Consequently, cases expressing MV_{129} or VV_{129} may be identified in the future, and it is possible that these may present with a different clinicopathologic phenotype compared with the cases seen to date. In addition, the recent discovery of transmission of vCJD via blood in three individuals has stirred up worries that blood-borne prion transmission, in combination with an unknown prevalence of vCJD-infected carriers, may lead to secondary transmission of host-adapted prions (Hewitt*, et al.*, 2006; Peden *et al.*,

2004). This could possibly result in a lengthening of the vCJD epidemic or, in the worst-case scenario, may cause vCJD to become endemic and self-sustained.

Since the BSE prion is distinct from all other prions known to date, except for the vCJD prion which it is casually linked to, an interesting question is raised: Was a particular conformation of bovine PrP^{Sc} selected for heat resistance during the rendering process and then reselected multiple times as cattle infected by ingesting prion-contaminated MBM were slaughtered and their offal rendered into more MBM? In this regard it is interesting to note that some atypical cases of prion disease have recently been dicovered in cattle (Baron & Biacabe, 2006; Baron *et al.*, 2006; Biacabe *et al.*, 2004; Buschmann *et al.*, 2004; Casalone*, et al.*, 2004). Could any of these possibly sporadic/spontaneous TSEs in cattle be the origin of the BSE prion? Future investigations will hopefully answer these questions.

Nor98

A number of atypical cases of scrapie were diagnosed in sheep in Norway for the first time in 1998 and subsequently designated Nor98 (Benestad*, et al.*, 2003). Similar atypical scrapie cases were also reported from other parts of Europe (De Bosschere *et al.*, 2004; Everest *et al.*, 2006; Onnasch *et al.*, 2004) including Sweden (Gavier-Widen *et al.*, 2004). Nor98 cases differ from classical scrapie and BSE in several features, including the pattern of PrP^{Sc} deposits and the neuroanatomical distribution of the histopathological lesions in the brain (Benestad*, et al.*, 2003). Other uncommon features are observed in the clinical presentation, epidemiology, and frequency of genotypes of sheep affected (Benestad*, et al.*, 2003; Gavier-Widen*, et al.*, 2004; Klingeborn*, et al.*, 2006b; Moum *et al.*, 2005). The differentiation between classical scrapie and Nor98 is based on these features and substantiated by the observation of the Nor98 western blot electrophoretic profile, typified by a fast migrating PK-resistant fragment (Benestad*, et al.*, 2003; Klingeborn*, et al.*, 2006b). PK-treated brain extracts from Nor98 cases demonstrate PrP fragments of sizes similar to full-length as well as a smaller fragment migrating to around 7 kDa (Klingeborn*, et al.*, 2006b). The smallest fragment seen in classical scrapie extracts is a C-terminal, PK-resistant peptide of around 19-21 kDa (Hayashi *et al.*, 2005; Hope *et al.*, 1999; Stahl *et al.*, 1993). However, brain extracts from subjects affected by certain types of Gerstmann-Sträussler-Scheinker disease (GSS) contain PrP-res fragments of around 11 to 7 kDa carrying ragged N- and C-termini (Ghetti *et al.*, 1996; Parchi *et al.*, 1998; Piccardo *et al.*, 1995; Piccardo *et al.*, 2001; Piccardo *et al.*, 1996; Tagliavini *et al.*, 2001; Tagliavini *et al.*, 1991). GSS is a prion disease with a genetic background with various mutations in the open reading frame of the prion protein gene (for a review see (Young *et al.*, 1999)).

Differently, in Nor98-affected sheep no mutations in the structural gene of PrP that can be correlated to the aberrant PK-resistant profile have been observed (Klingeborn*, et al.*, 2006b; Moum*, et al.*, 2005). Moum et al. recently sequenced the open reading frame (ORF) of *PRNP* in 38 Norwegian cases of Nor98 and an association with polymorphisms at codons 141 and 154 (ovine numbering) was found (Moum*, et al.*, 2005). In this aspect, the first six cases found in Sweden

(2003-2005) were homozygous for leucine at position 141 and did not display the association found in Norway. However, the genotype distribution at codons 136, 154 and 171 in five of the six cases resembled the pattern found among Nor98 cases in Norway in that ARQ and/or AHQ is always present while VRQ is missing. However, one case carried the $A_{136}R_{154}R_{171}/A_{136}R_{154}H_{171}$ genotype (Klingeborn*, et al.*, 2006b). The widespread geographical distribution, single cases in each affected flock and the lack of genetic evidence suggests that Nor98 could possibly be a spontaneous/sporadic prion disease. In this regard it is noteworthy that similar to the Nor98/GSS comparison, sporadic fatal insomnia (sFI) show all the hallmarks of familial fatal insomnia (FFI) but lack the D178N substitution that is always associated with FFI (Mastrianni *et al.*, 1999; Montagna*, et al.*, 2003; Pan *et al.*, 2001; Parchi *et al.*, 1999; Piao *et al.*, 2005; Scaravilli *et al.*, 2000; Watts, Balachandran & Westaway, 2006).

The likelihood that Nor98 cases were not genuine TSEs was excluded by the transmission of ten French atypical isolates and three Norwegian Nor98 cases to transgenic mice overexpressing ovine PrP ($V_{136}R_{154}Q_{171}$)(Le Dur *et al.*, 2005).

It is interesting to note that Nor98 affect sheep carrying genotypes which are considered to confer a relatively high or high resistance to scrapie infection (Benestad*, et al.*, 2003; Gavier-Widen*, et al.*, 2004; Klingeborn*, et al.*, 2006b; Moum*, et al.*, 2005).This would suggest that ARR/ARR sheep can no longer be considered as free of natural TSE infection. Furthemore, this finding puts serious strain on, at least to some extent, the foundation of the selective breeding programs implemented in several European Union member states (Anonymous, 1999; Anonymous, 2006; Arnold *et al.*, 2002; Baylis & McIntyre, 2004) and may call for a reassessment of consequences of this guiding principle in the long term. The discovery of Nor98 and other atypical scrapie strains could also give a possible explanantion to the existence of the VRQ haplotype. The existence of this haplotype has been perplexing because it is not known to bestow advantages to the host, on the contrary, it is frequently fatal for sheep exposed to classical scrapie. A haplotype of this sort would be expected to be eliminated from the population by natural selection, why is it not so? Perhaps the answer lies in the presence of these newly discovered atypical scrapie strains? Could it in fact be that the VRQ haplotype conferred resistance to past strains? If that is the case, it is a strong reason for maintaining a large diversity of PrP haplotypes in sheep populations. More information about this newly discovered type of TSE agent, its occurrence in countries free of scrapie or BSE disease, and its capability for intra- and inter-species transmission is needed, to be able to assess its implications in terms of veterinary public health.

CWD – an emerging prion disease

CWD was first recognized in 1967 as a clinical syndrome of unknown etiology among captive mule deer (*Odocoileus hemionus*) at wildlife research facilities in Colorado and Wyoming in the US (Watts, Balachandran & Westaway, 2006; Williams, 2003). It was recognized as a spongiform encephalopathy by histopathological examination of the CNS from affected animals in 1978

(Sigurdson & Miller, 2003; Williams & Young, 1980; Williams & Young, 1982). Biochemical and histopathological features have been described comprehensively and fall within the mainstream of prion diseases (Williams, 2005). There are only three species of *Cervidae* (the deer family) known to be susceptible to CWD in the field: mule deer, white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain Elk (*Cervus elaphus nelsoni*).

CWD have two remarkable features that call for attention. First, it is the only prion disease known to (naturally) affect free-ranging animals. Second, it is a disease where horizontal transmission is very common (Mathiason *et al.*, 2006; Miller, Wild & Williams, 1998; Miller *et al.*, 2000). Related to the second point, CWD can reach impressive attack rates in affected populations, around 30% in some local populations of deer and effectively 100% in captive research facilities (Williams, 2005), and is spreading across North America in both captive and free-ranging populations. This spread has made CWD a disease of considerable concern for the captive elk and deer industry. Even though only three species of the *Cervidae* are known be naturally infected at present, there are concerns of the CWD prion crossing the species barrier into new hosts, including humans (Anonymous, 2003; Mathiason*, et al.*, 2006; Raymond*, et al.*, 2000). If also moose (*Alces sp.*) can be naturally infected (Watts, Balachandran & Westaway, 2006; Williams, 2005), the scenario for CWD reaching new geographical areas are worrying.

The PrP gene

The PrP gene is highly conserved in mammals, and paralogues are present in turtles (Simonic *et al.*, 2000), amphibians (Strumbo *et al.*, 2001) and fish (Premzl *et al.*, 2004; Rivera-Milla, Stuermer & Malaga-Trillo, 2003). The complete open reading frame (ORF) of all known vertebrate PrP genes is situated within a single exon (Basler *et al.*, 1986; Gabriel *et al.*, 1992; Hsiao*, et al.*, 1989; Premzl*, et al.*, 2004; Prusiner, 1998; Westaway *et al.*, 1987; Windl *et al.*, 1995). The mouse, sheep, cattle, and rat PrP genes contain three exons with the ORFs in exon 3 (Saeki *et al.*, 1996; Westaway *et al.*, 1994a; Westaway *et al.*, 1991; Westaway *et al.*, 1994c; Yoshimoto *et al.*, 1992) which is analogous to exon 2 of the human and syrian hamster gene which both only contain 2 exons (Basler*, et al.*, 1986; Kretzschmar *et al.*, 1986b; Lee *et al.*, 1998; Makrinou, Collinge & Antoniou, 2002; Puckett *et al.*, 1991). PrP mRNA is expressed at high levels in neurons (Kretzschmar *et al.*, 1986a) but is also expressed in a variety of non-neural tissues throughout the body (Brown *et al.*, 1990; Han, Liu & Zhao, 2006; Makrinou, Collinge & Antoniou, 2002).

Classical genetic analysis identified scrapie incubation (Sinc)/prion incubation (Prn-i) as the major gene controlling mouse scrapie incubation time. It is important to acknowledge that the Sinc/Prn-i gene and the PrP gene (*Prnp*) are one and the same (Carlson *et al.*, 1986; DeArmond & Prusiner, 2003; Moore *et al.*, 1998; Westaway*, et al.*, 1987).

The doppelgänger of PrP

16 kb downstream of the PrP gene, an ORF is present that encodes a protein, which shares significant homology with PrP^C (Moore *et al.*, 1999). It is probably the result of a gene duplication of a single ancestral gene which gave rise to *Prnp* and *Prnd*, as it is highly homologous to the C-terminal two-thirds of PrP. The protein of 179 residues was named Dpl for "downstream of the *Prnp* locus" (or "doppel") (Moore*, et al.*, 1999). The *Prnd* gene coding for Dpl is evolutionarily conserved from humans to sheep and cattle, suggesting an important function of Dpl (Tranulis *et al.*, 2001). In fact, the absence of Dpl causes male sterility in mice (Behrens *et al.*, 2002). Spermatozoa from Dpl-deficient mice seem to be incapable of undergoing the normal acrosome reaction that is needed to penetrate the *zona pellucida* of the ovum. Thus, mice lacking *Prnd* identify Dpl as a crucial regulator of male gametogenesis. At present, the experimental data is inconclusive in regard to the possible involvement of Dpl in prion disease pathogenesis (Aguzzi & Polymenidou, 2004; Rossi *et al.*, 2001).

Cellular biology of PrP^C

Investigating the biosynthesis, posttranslational processing, cellular localization, and trafficking of PrP^C and PrP^{Sc} allows examination of the properties of PrP in the setting of native cellular structures. The function of PrP^C and co-factors that are likely to be critical in mediating the efficient conversion of PrP^C into PrP^{Sc} can only be elucidated in detail in a cell culture model system.

Expression and function of PrP^C

PrP^C is a glycoprotein, normally attached to the surface of neurons, especially synaptic membranes, and glia cells of the brain and spinal cord in all mammals via a glycophosphatidylinositol (GPI) anchor (Moser *et al.*, 1995; Stahl *et al.*, 1987) (see below Figure 3). The expression pattern of PrP^C is diverse and developmentally regulated in skeletal muscle, kidney, heart, secondary lymphoid organs and the CNS, suggesting a wide-ranging and conserved function of the protein (Aguzzi & Polymenidou, 2004; Bendheim *et al.*, 1992; Caughey, Race & Chesebro, 1988; Dodelet & Cashman, 1998; Ford *et al.*, 2002; Harris, Lele & Snider, 1993; Manson *et al.*, 1992; Miele *et al.*, 2003; Moser*, et al.*, 1995).

Establishing the physiological role of PrP^C may be important for understanding of the disease state, since the protein may fail to carry out its normal function when it is converted into the PrP^{Sc} isoform. In the words uttered by the Nobel laureate Dr Kurt Wüthrich at the 2003 Prion Conference in Munich: "PrP^C is the key" in response to the endless number of experiments done exclusively on the disease-associated isoform, PrP^{Sc}. To add to the conundrum of PrP, mice devoid of PrP do not display any defects (Bueler *et al.*, 1992), not even when ablation is induced postnatally in neurons (Mallucci *et al.*, 2002). In fact, the only definite phenotype of $PRNP^{0/0}$ mice is their resistance to prion infection (Bueler*, et al.*, 1993)!

Its expression in most tissues and its amino acid (aa) conservation throughout evolution would argue for PrP^C having a fundamental function. However, the function of PrP^C is still enigmatic. Its localization on the extracellular face of the cell membrane would be consistent with roles in cell adhesion and recognition, ligand uptake, or transmembrane signalling. Some of the many functions that have been attributed to PrP^C relates to immunoregulation, signal transduction, copper binding, synaptic transmission, superoxide dismutase activity, cell adhesion, induction of apoptosis or protection against apoptosis to name but a few (Brown *et al.*, 1997; Brown *et al.*, 1999; Kurschner & Morgan, 1995; Mouillet-Richard *et al.*, 2000; Sakudo *et al.*, 2003; Schmitt-Ulms *et al.*, 2001). PrP^C is expressed on long-term, re-populating hematopoietic stem cells (Zhang *et al.*, 2006) and it positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis (Steele *et al.*, 2006). Recently PrP^C has also been implicated in cell-cell communication via exosomal release pathways (Fevrier *et al.*, 2004) and long-term memory mechanisms (Papassotiropoulos *et al.*, 2005).

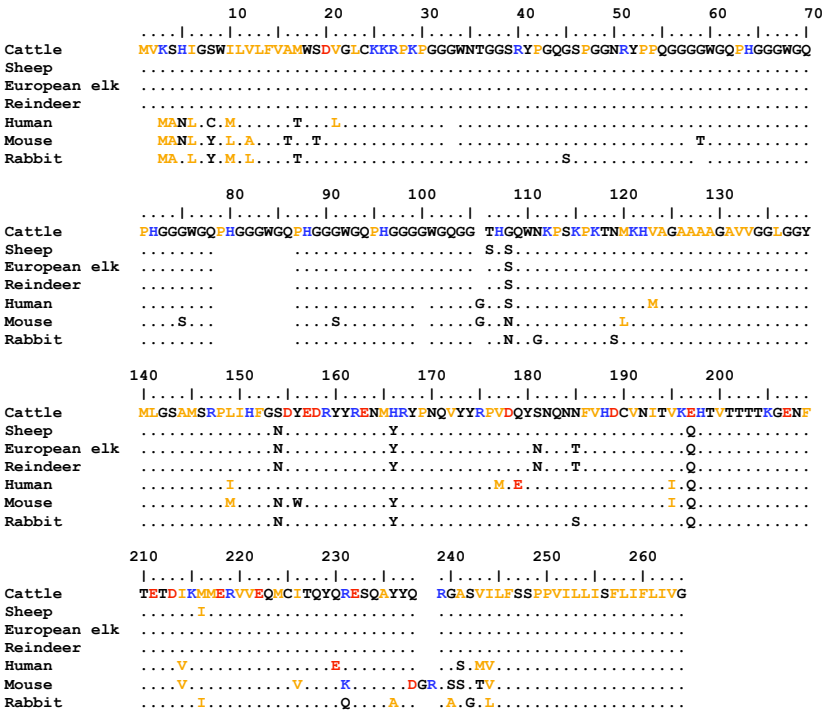

Figure 1. Amino acid alignment of PrP from seven species. Protein accession nos. in the NCBI database (http://www.ncbi.nlm.nih.gov/) are P10279 (Cattle), NP_001009481 (Sheep), AAT77255 (European elk), AAT77253 (Reindeer), P04156 (Human), NP_035300 (Mouse), AAC48697 (Rabbit). Amino acid numbering is according to boPrP (6OR). The aa residues have been coloured according to their polarity characteristics as acidic (red), basic (blue), hydrophobic (light orange), and uncharged polar (black), respectively. The alignment was done using ClustalW and the figure was generated in BioEdit (v. 7.0.5).

Biosynthesis of PrP^C

The PrP encoded by the mammalian PrP gene comprise approximately 250 aa (Fig. 1) and contains a number of discrete domains. It contains an N-terminal signal peptide, a series of five proline- and glycine-rich octapeptide repeats, a central hydrophobic region that is highly conserved, and a C-terminal hydrophobic portion that functions as a signal for GPI anchor addition (Figure 2). The human PrP gene encodes 253 aa and the sheep 256 aa. Interestingly, in cattle approximately 90% is homozygous for a PrP with 6 octapeptide repeats (6OR) and the remaining 10% heterozygous for PrP with 5 and 6 repeats (Heaton *et al.*, 2003; Jeong *et al.*, 2005; Nakamitsu *et al.*, 2006; Sander *et al.*, 2004). Cattle homozygous for 5 octapeptide repeats have been found, but are very rare (Nakamitsu*, et al.*, 2006). Strictly speaking, in cattle the octapeptide repeat region consists of 1 nonapeptide followed by 4(3) octapeptides followed by 1 nonapeptide:

nonarepeat 1: aa 54 - 62	(PQGGGGWGQ)
octarepeat 1: aa 63 - 70	(PHGGGWGQ)
octarepeat 2: aa 71 - 78	(PHGGGWGQ)
octarepeat 3: aa 79 - 86	(PHGGGWGQ)
octarepeat 4: aa 87 - 94	(PHGGGWGQ)
nonarepeat 2: aa 95 - 103	(PHGGGGWGQ)

Amino acid numbering is according to the 6OR bovine PrP (P10279 in the NCBI database) sequence which consists of 264 aa. The four octarepeats are identical while the two nonarepeats differ at one position.

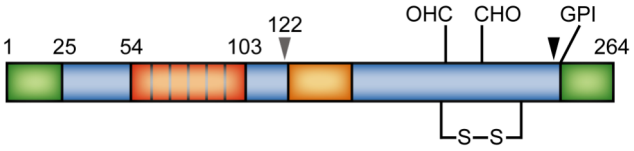

Figure 2. Schematic representation of the boPrP primary translational product. Numbering in the figure is according to the 6OR boPrP. N-terminal signal sequence (1–24, green box), six octarepeats (54–103, red boxes), hydrophobic region (123–150, gold box), C-terminal signal sequence for GPI attachment (242–264, green box), N-linked glycosylation at N_{192} and N_{208} (CHO), disulphide bond between C_{190} and C_{225} (S-S), and GPI-anchor is attached at aa 241. The cleavage site between K_{121} and H_{122} generating the N1 and C1 fragments is indicated by a grey arrowhead and the putative cleavage site for shedding of PrP in the region 238-QRGA-241 is indicated by a black arrowhead.

PrP^C is synthesized in the rough endoplasmic reticulum (ER) and transits the Golgi on its way to the surface, similar to other membrane proteins. Throughout the biosynthesis of PrP^C, it undergoes a number of different posttranslational modifications. These modifications include cleavage of the N-terminal signal peptide, addition of N-linked oligosaccharide chains at two sites (N_{192} and N_{208}), formation of a single disulfide bond (C_{190} and C_{225}), and addition of a GPI anchor

(Haraguchi *et al.*, 1989; Harris, 1999; Stahl, *et al.*, 1987; Turk *et al.*, 1988; Zhao *et al.*, 2006). In the ER N-linked oligosaccharides of high-mannose type are added that are sensitive to endoglycosidase H treatment and the GPI-anchor is added after cleavage of the C-terminal hydrophobic portion. Next, these oligosaccharides are modified in the Golgi to complex-type sugar chains that contain sialic acid and gain resistance to endoglycosidase H (Caughey *et al.*, 1989; Zhao, *et al.*, 2006).

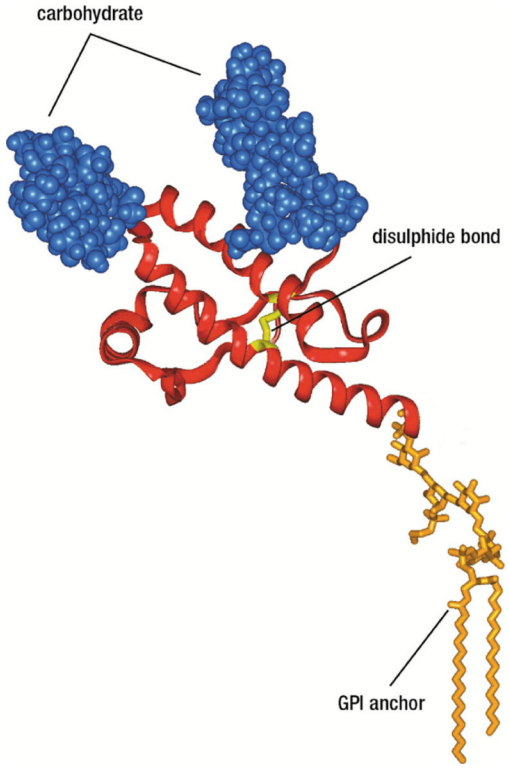

Figure 3. Model of glycosylated huPrP indicating positions of N-linked glycans (in blue spacefill), the single disulphide bond joining helices 2 and 3 (yellow bond), and the GPI-anchor that inserts into the outer leaflet of the plasma membrane. From (Collinge, 2001).

When both asparagine-linked consensus sites are glycosylated, the diversity of oligosaccharide structures yields over 400 different forms of PrP (Endo *et al.*, 1989). In combination with the six most commonly occurring forms of the GPI anchor found on PrP (Stahl *et al.*, 1992) the diversity of distinctly modified PrP molecules is vast. The size of the complex oligosaccharides and the GPI anchor moiety in relation to the polypetide is considerable as can be seen in Figure 3.

Molecules with either one or both N-glycosylation attachment sites mutated display a number of biochemical characteristics of PrP^{Sc} (Rogers *et al.*, 1990). A conclusion of these experiments could be that wild-type PrP^{C} has an inherent propensity to take on some properties of PrP^{Sc} in the course of its normal

conformational maturation but that N-linked oligosaccharides safeguard against this transformation. The observation that different prion strains exhibit characteristic patterns of glycosylation could be related to this inherent propensity of PrP (Collinge *et al.*, 1996; DeArmond *et al.*, 1997; Kascsak *et al.*, 1986; Monari *et al.*, 1994; Parchi *et al.*, 1996).

Posttranslational cleavage of PrP^C

Work with transfected cell lines, brain tissue, cerebrospinal fluid, blood, and tissue from a number of organs, have shown that PrP^C is subjected to two posttranslational cleavages during the course of its normal metabolism (Borchelt *et al.*, 1993; Caughey*, et al.*, 1989; Caughey *et al.*, 1988; Chen *et al.*, 1995; Harris *et al.*, 1993; Jimenez-Huete *et al.*, 1998; Klingeborn *et al.*, 2006a; Perini, Frangione & Prelli, 1996; Tagliavini *et al.*, 1992; Taraboulos *et al.*, 1995; Tveit *et al.*, 2005; Vincent *et al.*, 2000; Vincent *et al.*, 2001; Volkel *et al.*, 2001; Yadavalli *et al.*, 2004; Zhao*, et al.*, 2006). The first cleavage occurs within a segment of hydrophobic aa (huPrP 112-139; boPrP 123-150) that is almost completely conserved in all vertebrate PrPs (see Figure 1 and (Schatzl *et al.*, 1995)). The resulting N-terminal fragment, N1, is recovered in the extracellular space and the C-terminal fragment (C1) with alternative N-termini at H_{111} or M/V_{112} (huPrP numbering; K_{121}/H_{122} in boPrP) remains attached to the cell surface via its GPI anchor (Chen*, et al.*, 1995; Harris*, et al.*, 1993; Vincent*, et al.*, 2000; Vincent*, et al.*, 2001; Zhao*, et al.*, 2006). Our data indicate that this cleavage, denoted C1 cleavage, occurs beyond the trans-golgi network (TGN) but prior to or very soon after arrival at the cell surface (Zhao*, et al.*, 2006). Similar observations have been made by other groups (Taraboulos*, et al.*, 1995; Tveit*, et al.*, 2005), while some groups suggest that it occurs in an endocytic compartment following endocytosis (Harris*, et al.*, 1993; Shyng, Huber & Harris, 1993). Differences in the protease activity in subcellular compartments could be the reason for the diverging observations of proteolytic processing between cell lines.

The second cleavage occurs at the extreme C-terminal end, probably only 3 aa from the GPI anchor by a zinc metalloprotease(s) and results in the shedding of C1 into the extracellular medium (Borchelt*, et al.*, 1993; Parkin *et al.*, 2004; Stahl *et al.*, 1990; Zhao*, et al.*, 2006). The time course of this second cleavage suggests that it possibly takes place during cycling between the cell surface and an endocytic compartment with a transit time of approximately 60 min (Klingeborn*, et al.*, 2006a; Shyng, Huber & Harris, 1993; Zhao*, et al.*, 2006). Interestingly, in some cell lines where this specific protease activity is low in these compartments, a minor release by a cleavage within the GPI anchor have been reported (Harris*, et al.*, 1993; Shyng, Huber & Harris, 1993). This cleavage is affected in a dose-dependent manner by the amount of fetal calf serum (FCS) included in the cell culture medium, implicating an exogenous phospholipase (Harris*, et al.*, 1993; Klingeborn*, et al.*, 2006a; Parizek *et al.*, 2001; Shyng, Huber & Harris, 1993). Supporting this notion, phospholipase-mediated cleavage cannot be detected in the absence of FCS in our hands (Klingeborn*, et al.*, 2006a) and was recently reported to only be detected after induction by lipid raft-disrupting agents (Parkin*, et al.*,

2004) suggesting that this type of cleavage is non-constitutive and possibly not present under physiological conditions.

The C1 cleavage is very fast, so at steady-state a major fraction of PrP^C will be in the form of C1, and this is also supported by observations both *in vitro* and *in vivo* in many species (Chen, *et al.*, 1995; Jimenez-Huete, *et al.*, 1998; Klingeborn, *et al.*, 2006a; Klingeborn, *et al.*, 2006b; Liu *et al.*, 2001; Takekida *et al.*, 2002; Tveit, *et al.*, 2005; Zhao, *et al.*, 2006), see also Figure 4. Both of these proteolytic processing events occur relatively quickly in relation to the half-life of the protein. C1 is detected as soon as 5-20 min after translation and the soluble form, C1-S, is recovered in the extracellular medium 60-75 min after translation. The halflife of C1 is long, probably in excess of 24 hours (Klingeborn, *et al.*, 2006a). Interestingly, in some cell lines a more complete degradation of PrP, almost completely without C1 cleavage and shedding, have been described (Caughey, *et al.*, 1989; Zhao, *et al.*, 2006). The underlying cause for this divergence in PrP metabolism is not known at this stage.

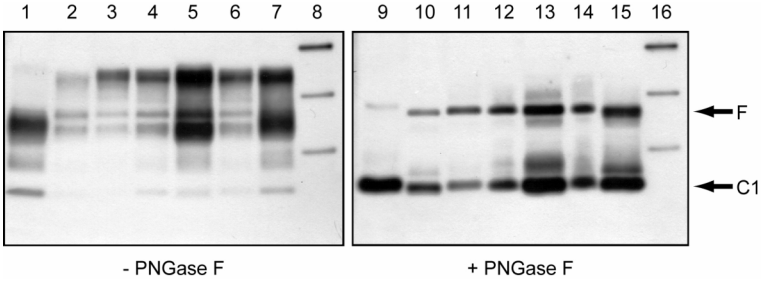

Figure 4. Western immunoblotting of PrP^C (mAb 6H4) in brain homogenates from a number of species demonstrating the C1 fragment. Cattle (lanes 1 and 9), sheep (lanes 2 and 10), European elk (lanes 3 and 11), roe deer (lanes 4 and 12), reindeer (lanes 5 and 13), fallow deer (lanes 6 and 14), red deer (lanes 7 and 15), and molecular mass markers (lanes 8 and 16; 20, 30 and 40 kDa bands are visible). Removal of oligosaccharides by treatment with PNGase F and positions of full length (F) and the C1 fragment (C1) of PrP^C are indicated. (Simonsson, M., Klingeborn, M., and Linné, T., unpublished)

The physiological importance of these cleavages is unknown. Similar to polypeptide growth factors that are released by cleavage of membrane-bound precursors, the N1 could serve as a biologically active ligand (Nakada-Tsukui, Watanabe & Kobayashi, 1999; Parkin *et al.*, 2002; Zheng *et al.*, 2004). In fact, some of the proteases implicated in the C1 cleavage releases a number of biologically active ligands (Parkin, *et al.*, 2004; Vincent, *et al.*, 2000; Vincent, *et al.*, 2001; Zhao, *et al.*, 2006). PrP^C could possibly function as a cell surface receptor and the cleavages would in that case correspond to mechanisms of receptor down-regulation.

Exosome-mediated release of PrP

In addition to protease-mediated shedding of PrP, another constitutive pathway of release of PrP have recently been discovered. This type of release is exosome-

mediated and is responsible for <1% of the PrP recovered from the extracellular medium (Fevrier, *et al.*, 2004; Klingeborn, *et al.*, 2006a; Leblanc *et al.*, 2006; Rajendran *et al.*, 2006). Exosomes are 50- to 90-nm vesicles of endosomal origin that are released by many cell types into the extracellular environment upon fusion of endosomes known as multivesicular bodies (MVBs), with the cell surface (Stoorvogel *et al.*, 2002; Thery, Zitvogel & Amigorena, 2002). They are thought to be play an important role in intercellular communication (Stoorvogel, *et al.*, 2002; van Niel *et al.*, 2006) and further studies of this pathway of PrP^{C} release could reveal interesting details about functional aspects of PrP^{C}. It has been reported recently that purified exosomes from scrapie-infected cells could infect both cell cultures and bioassay mice very efficiently (Fevrier, *et al.*, 2004). Since only very few molecules of PrP^{Sc} are needed to transmit prion infection (Silveira *et al.*, 2005) even a minute pathway of release such as the exosome-mediated could still be important for cell-to-cell spreading of prion infection. The importance of the exosomal release of PrP^{Sc} for the spread of prion infection remains to be investigated.

Cellular biology and propagation of PrP^{Sc}

In the case of conventional virus diseases, experimental analysis depends largely on the ability to grow the infectious agent in cultured cells, and the same has been true for prion diseases. PrP^{Sc} is produced by several cell types following prion infection *in vivo*, including neurons, astrocytes, and lymphoreticular cells (Blattler *et al.*, 1997; DeArmond *et al.*, 1987; Diedrich *et al.*, 1991; Raymond, *et al.*, 1997). It has however been quite difficult to achieve cell lines stably infected with prions. Most of the cell lines that are stably infected are neuronally derived but some of non-neuronal origin have also been established (Archer *et al.*, 2004; Butler *et al.*, 1988; Clarke & Haig, 1970; Race, Fadness & Chesebro, 1987; Raymond *et al.*, 2006; Sabuncu *et al.*, 2003; Schatzl *et al.*, 1997; Vorberg *et al.*, 2004). These stably infected cells continuously produce low levels of PrP^{Sc}, which can be recognized by its proteinase resistance and detergent insolubility and by its infectivity in animal bioassays.

Properties of stably prion infected cell lines

A crucial property of a cell line which enable it to propagate prions is similarity between the sequence of the endogenous PrP^{C} and that of the donor PrP^{Sc} (Weissmann, 2004). Even small differences not only prevent conversion of the mismatched PrP^{C} (Priola & Chesebro, 1995) but also have a dominant-negative effect on the conversion of matching PrP^{C} in the same cell (Kaneko *et al.*, 1997). Stably infected cells do not display any obvious cytopathology, which might seem surprising. However, this finding is probably connected with the properties that enable stable prion infection and is explained by the "dynamic susceptibility model" proposed by Weissmann (Weissmann, 2004). This model suggests that the ability of a cell to propagate prions, all genetic characteristics being alike, rely on the ratio of prion synthesis to degradation. The rate of prion formation must equal or surpass twice that of its degradation, or the infected condition will be eliminated at some time after exposure to exogenous prions. This is a consequence of

infectivity per cell being halved after each cell division. However, a rate of formation that is more than twice that of degradation will result in a steady accumulation of PrP^{Sc} that might eventually lead to cell death. Therefore, maintenance of a precise equilibrium of synthesis versus degradation is crucial to attain persistent infection. Interestingly, cell lines that are susceptible to certain prion strains might not be susceptible to other strains and this strain susceptibility differs between cell lines. The underlying mechanisms for these observations are not known at present.

Formation of PrP^{Sc} in cultured cells

It has been established that PrP traverses the mid-Golgi stack before acquiring protease resistance. About 1 h after the formation of PrP^{Sc}, its N-terminus is removed by a proteolytic process, resulting in a fragment denoted C2, that is inhibited by ammonium chloride, chloroquine, leupeptin, calpain inhibitors and monensin, arguing that it is an event occurring in the late endocytic pathway or lysosome (Caughey *et al.*, 1991; Taraboulos *et al.*, 1992; Yadavalli, *et al.*, 2004). These results also suggest that the ER is unfit for the synthesis of PrP^{Sc}. The C2 fragment is identical with the fragment produced by PK-treatment of PrP^{Sc}, PrP 27-30 (Chen, *et al.*, 1995; Yadavalli, *et al.*, 2004). Furthermore, surface iodination of infected cells results in incorporation of radiolabel into PrP^{Sc} after a chase period, arguing that PrP molecules transit the plasma membrane prior to conversion into PrP^{Sc} (Caughey, *et al.*, 1991). Treatment of cells with phosphatidylinositol-specific phospholipase C (PIPLC) or proteases inhibits the production of PrP^{Sc}, probably by removal of the PrP^{C} substrate from the cell surface (Borchelt, Taraboulos & Prusiner, 1992; Caughey & Raymond, 1991). The formation of PrP^{Sc} is also diminished by lovastatin-mediated depletion of cellular cholesterol but insensitive to ammonium chloride. Thus, it seems likely that the formation of PrP^{Sc} occur within a non-acidic compartment bound by cholesterol-rich membranes, possibly glycolipid-rich microdomains, where the metabolic fate of PrP^{C} is determined (Taraboulos, *et al.*, 1995). Presumably, the conversion of PrP to the protease-resistant state occurs in the plasma membrane or along the endocytic pathway before PrP^{Sc} is exposed to endosomal and lysosomal proteases (Caughey, *et al.*, 1991; Chen, *et al.*, 1995; Taraboulos, *et al.*, 1992; Yadavalli, *et al.*, 2004).

In scrapie-infected Syrian hamster (SHa) brain full-length PrP^{Sc} is also detected, indicating that some but not all PrP^{Sc} molecules accumulating in the brain during scrapie lose their N-terminus. Thus, lysosomal trimming is not an obligatory step in PrP^{Sc} synthesis; instead, the lysosomal accumulation of PrP^{Sc} may reflect ineffective attempts of cells to degrade unwanted proteins (Taraboulos, *et al.*, 1992). Because the processing of PrP^{C} appears to be mainly nonlysosomal (Klingeborn, *et al.*, 2006a; Taraboulos, *et al.*, 1995; Yadavalli, *et al.*, 2004; Zhao, *et al.*, 2006), PrP^{Sc} must get some sorting signal that targets it to secondary lysosomes. Investigating the nature of such a signal may give important information regarding the structural difference between the PrP isoforms. One possibility is that aggregation of PrP^{Sc} causes its endocytosis.

Once formed, PrP^{Sc} appears to be metabolically stable for 24 to 48 h, in contrast to PrP^{C}, which turns over with a half-life of 4 to 6 h (Borchelt *et al.*, 1990; Borchelt, Taraboulos & Prusiner, 1992; Caughey & Raymond, 1991). Only a minority of PrP^{C} molecules, around 5%, are converted to PrP^{Sc} in infected cells, with the remainder being processed and degraded by pathways similar to those found in uninfected cells (Borchelt, *et al.*, 1990; Taraboulos *et al.*, 1990; Taraboulos, *et al.*, 1995).

Propagation of PrP^{Sc}

There are currently two models favoured to explain the mechanistic aspects of the conversion of PrP^{C} to PrP^{Sc} (Figure 5). The "template-directed refolding model" (also known as the "monomer-directed heterodimer model") predicates that PrP^{C} unfolds to some extent and refolds under the influence of a PrP^{Sc} molecule that acts as a template and possibly along with a hypothetical molecular chaperone (Kaneko, *et al.*, 1997; Prusiner, Torchia & Westaway, 1991; Telling *et al.*, 1995). In this case, the kinetic barrier between PrP^{C} and PrP^{Sc} is overcome by the catalytic action of PrP^{Sc}, possibly in conjunction with the chaperone. Experimental evidence is compatible with this theory, however no positive proof in its favour has come forward to date.

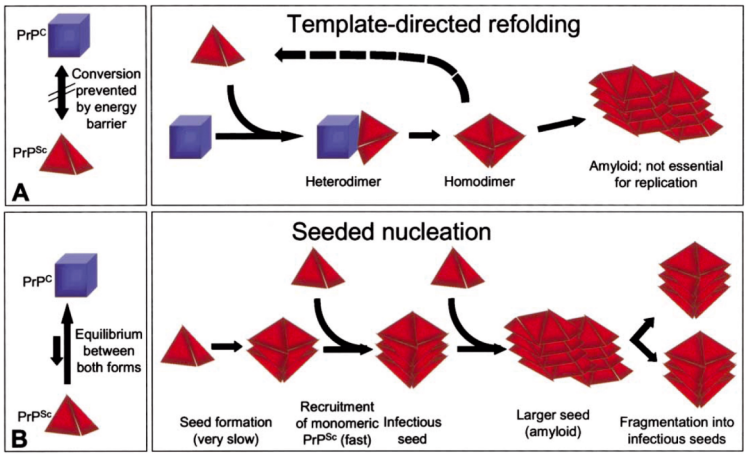

Figure 5. Models for the conformational conversion of PrP^{C} into PrP^{Sc}. (A) A schematic representation of the "template-directed refolding model". (B) Representation of the "seeded nucleation model". Details are described in the text. Figure from Aguzzi & Polymenidou (2004).

The "seeded nucleation model" (also known as the "nucleation-dependent polymerization model") on the other hand, postulates that PrP^{C} is in equilibrium with PrP^{Sc} (or a precursor thereof), with the equilibrium heavily in favour of PrP^{C}, and that PrP^{Sc} is only stabilized when it adds to a crystal-like seed of PrP^{Sc} (Come, Fraser & Lansbury, 1993; Jarrett & Lansbury, 1993). From this model follows that monomer addition will proceed rapidly as soon as a seed is present. To explain the exponential increase of PrP^{Sc} during infection, fragmentation of aggregates must

occur occasionally. Fascinatingly, it also follows that minute amounts of PrP^{Sc} is present at any time in a normal organism. If this was the case, it would be impossible for PrP^{Sc} to represent the infectious agent since it would be ubiquitous. The seeded nucleation model suggest that the infectious agent would consist of a highly ordered aggregate of PrP^{Sc} molecules. The aggregated condition would be an inherent feature of infectivity, i.e. monomeric PrP^{Sc} would be harmless but might be inclined to incorporate into PrP^{Sc} aggregates. These predictions have in fact been confirmed by the recent findings by Silveira and colleagues, who found that the most infectious prion protein particles were non-fibrillar particles of 14-28 PrP molecules (Silveira*, et al.*, 2005). Furthermore they found that the infectivity was substantially lower in large fibrils and virtually absent in oligomers of five or less PrP molecules.

In the case of yeast prions, as discussed below (*Prion strains*), experimental data strongly support the "seeded-nucleation model", and this also applies to mammalian prions as recently reported (Caughey *et al.*, 1997; Harper & Lansbury, 1997; Jones & Surewicz, 2005; Silveira*, et al.*, 2005; Surewicz, Jones & Apetri, 2006; Vanik, Surewicz & Surewicz, 2004). As discussed below regarding the "protein misfolding cyclic amplification" (PMCA) technique, the data available with the use of this method also strongly support the "seeded-nucleation model" of prion replication (Soto, Estrada & Castilla, 2006).

Prion strains

An extraordinary phenomenon is the existence of discrete prion strains, originally typified by the incubation time and neuropathology they elicit in a certain host (Bruce, 2003). As distinct mammalian prion strains can be propagated indefinitely in hosts homozygous for the PrP gene, the protein-only hypothesis stipulates that the strain-specific features must be enciphered in some property of the pathogenic PrP other than its amino acid sequence, such as posttranslational modification or conformation. Different strains are frequently associated with PrP^{Sc} species differing in physical features such as susceptibility to PK digestion, electrophoretic mobility following PK treatment reflecting a diversity of amino-termini as a result of different cleavage sites, stability toward denaturing agents, or the proportions of di-, mono-, and unglycosylated forms.

"Conformational templating" at the protein level was first demonstrated by showing that when radioactively labelled PrP^{C} was incubated with PrP^{Sc} derived from strains with different electrophoretic mobilities of their PK-resistant core fragment, the radioactive conversion product manifested the strain-specific attributes of the PrP^{Sc} template (Caughey*, et al.*, 1999). Experimental approaches with yeast [PSI^{+}] prions irrefutably established that different strain characteristics are associated with distinct fibrillar conformations of the Sup35 protein (King & Diaz-Avalos, 2004; Sparrer*, et al.*, 2000; Tanaka*, et al.*, 2004). In one approach, fibrous aggregates of Sup35 prion domains from three distinct [PSI^{+}] yeast strains were isolated, respectively. Preparations of bacterially expressed, tagged sup35 prion domains were seeded with these aggregates and the resulting fibers were introduced into yeast, where they evoke the [PSI^{+}] state characteristic for the

donor lines (King & Diaz-Avalos, 2004). The same conclusion was drawn from the results of another experimental approach: at different temperatures, amyloids formed *de novo* from the Sup35 prion domain adopted discrete, stably propagating conformations, as characterized by thermal stability and electron paramagnetic resonance spectroscopy, and infection of yeast with these amyloids led to distinct [PSI^{+}] strains (Tanaka*, et al.*, 2004).

With regard to mammalian prion strains, even though recombinantly expressed PrP (rPrP), both truncated and full-length, can be converted into fibrillar amyloid forms that can be propagated by seeding (Bocharova *et al.*, 2005; Jones & Surewicz, 2005), the infectivity, if any, of such preparations is low (Legname*, et al.*, 2004). Correlation of structurally diverse fibrils with distinct prion strains has not been achieved to date, but interesting insights concerning their seeding properties have been acquired (Jones & Surewicz, 2005; Legname*, et al.*, 2005; Surewicz, Jones & Apetri, 2006).

Species and strain barriers

Inter-species prion transmission is often restricted by a "transmission barrier" that is typified by prolonged incubation time, that the attack rate is incomplete, or that clinical disease is lacking. In spite of absence of clinical signs, prion replication, PrP^{Sc} accumulation, and histopathological changes may occur late after infection (Hill & Collinge, 2003). Incubation time, is not a straightforward function of prion or PrP^{Sc} accumulation but is influenced by the prion strain, the site of inoculation within the brain, and genetic determinants of the host other than the PrP gene. As judged by incubation time, transmission of prions from one species to another is typically less efficient than intra-species transmission, a phenomenon accredited to a "species barrier". Subsequent transmissions into individuals of the recipient species usually lead to a shorter incubation time than on primary passage. Prions therefore seem to "adapt" to the new host. The transmission barrier between species is ascribed to a mismatch between the amino acid sequence of the donor PrP^{Sc} and the recipient PrP^{C}, and in many cases substitution of the PrP gene of the mouse by that of the prion donor eliminates the barrier. "Adaptation" is due to the formation of PrP^{Sc} consisting of the host PrP (Prusiner*, et al.*, 1998). Even a single aa change in the PrP of the recipient can bring about a radical change in incubation time (Manson *et al.*, 1999) or even practically result in resistance to disease (dominant-negative mutations; (Perrier *et al.*, 2002)). Correspondingly, naturally occurring polymorphisms of the PrP gene, especially position 129 of the human PrP, have a major effect on susceptibility to sporadic and variant CJD (Ward *et al.*, 2003).

Different mouse prion strains, generated in mice with the same PrP gene, display different incubation times in a specific mouse line and this is also the case with human-derived vCJD and sCJD prions transmitted to mice (Korth *et al.*, 2003). This establishes that properties other than the PrP sequence or the host genotype, most likely the conformation of the PrP*, also are involved in dictating incubation times. Longer incubation times and low attack rates are credited to a "strain barrier". The strain and the species barrier most probably have a similar origin,

that is the inability of PrP^C to take on a conformation appropriate for efficient seed propagation. The difference between the two concepts is that for the "strain barrier" conformational space is intrinsic to one particular PrP sequence while for the "species barrier" conformational space is co-determined by variation in aa sequence.

The pathogenic mechanism

Prions multiply to high titers in the brain and in some hosts in spleen, albeit to a lower level. Spongiform degeneration, astrocytosis, and neuronal cell death accompany prion multiplication in the CNS. The pathogenic mechanism is still unclear, but is not due to the depletion of PrP^C since PrP gene knock out mice, whether inborn or induced post-natally, remain healthy (Bueler, *et al.*, 1992; Mallucci, *et al.*, 2002). PrP^{Sc} accumulates in the CNS and is in some instances deposited as an amyloid and has therefore been indicted as the toxic entity causing neuronal apoptosis and giving rise to disease (DeArmond & Prusiner, 2003). A number of experiments have shown that neurons devoid of PrP^C are not damaged by PrP^{Sc} (Brandner *et al.*, 1996; Bueler *et al.*, 1994). An interesting finding in this aspect is that when the neuronal expression of PrP in mice was prevented by conditional knock out 7-8 weeks after intracerebral inoculation, vast amounts of infectivity and PrP^{Sc} accumulated in astrocytes without exhibiting clinical symptoms or evidence of neuronal damage (Mallucci *et al.*, 2003). A toxic function gain by a PrP isoform that is different from PrP^{Sc} is therefore a definite possibility. This PrP isoform could be congruent with PrP* or yet another isoform distinct from PrP^C and PrP^{Sc}. Further work is needed to understand the biochemical pathways leading to pathogenicity, whether triggered by PrP^{Sc}, PrP* or some other isoform of PrP.

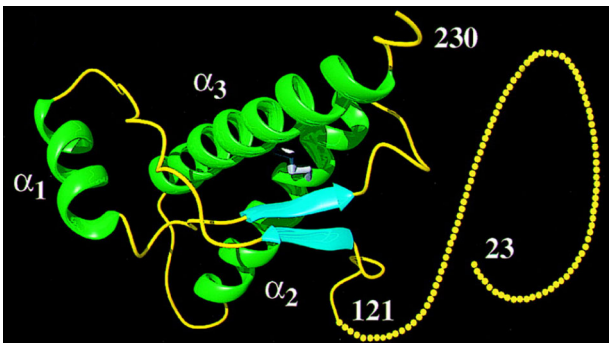

Figure 6. Structure of $boPrP^C$ determined by NMR. Helices are green, β-strands are cyan, segments with nonregular secondary structure within the C-terminal domain are yellow, and the flexibly disordered ''tail'' of residues 23–121 is represented by 108 yellow dots, each of which represents a residue of the tail (the numeration for huPrP is used). Adapted from Lopez Garcia *et al.* (2000).

Conformations of PrP

The three-dimensional structure of PrP is highly conserved between species (Wuthrich & Riek, 2001). The mature boPrP^{C} (boPrP aa 25-241) include an N-terminally flexible disordered "tail" comprising aa 25-135, a globular domain extending from residue 136-238, and a short flexible chain end of aa 239-241, respectively (Figure 6). The globular domain contains three alpha-helices comprising the residues 155-165, 184-205, and 211-237, and a short anti-parallell beta-sheet comprising the residues 139-142 and 172-175 (Lopez Garcia *et al.*, 2000; Zahn *et al.*, 2000). Helices 2 and 3 are linked by the only disulfide bond in boPrP (C_{190} and C_{225}).

The hydrophobicity of PrP^{Sc}, which is reflected partly by its detergent-insolubility, have hampered attempts to determine the tertiary structure of PrP^{Sc} by nucleomagnetic resonance (NMR) imaging. Because of the tendency of PrP^{Sc} to form large, heterogenous aggregates, also analysis by high-resolution X-ray crystallographic techniques have been difficult (Harris & True, 2006). It would thus seem unlikely that monomeric forms of PrP^{Sc} exist. However, it is clear that PrP^{Sc} have a much higher proportion of beta sheets than PrP^{C} (45% compared to 3%), as analysed by Fourier transform infrared spectroscopic methods (Pan *et al.*, 1993; Prusiner, 1998) . A model of PrP^{Sc} based on electron microscopic analysis of two-dimensional crystals of PrP 27-30 in combination with theoretical considerations revealed a stacked, left-handed β-helical structure (Govaerts *et al.*, 2004; Wille *et al.*, 2002) (Figure 7). Based on computer modelling or on structural analysis of recombinant PrP or on synthetic PrP peptides that have been induced to take on β-rich conformations, other models have also been suggested (Lee & Eisenberg, 2003).

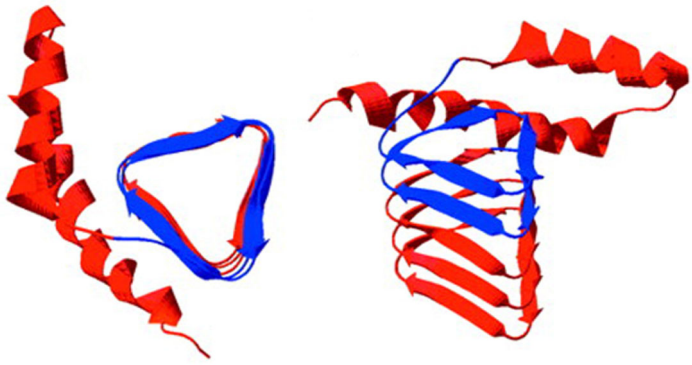

Figure 7. β-helical model of PrP 27-30. (Left and right) Top and side views, respectively, of PrP 27-30 modelled with a left-handed β-helix. Modified from Wille *et al.* (2002).

Proof of principle – the protein-only hypothesis revisited

Final proof that the prion comprise only protein would be achieved if pure PrP^{C} or, even better, recombinant PrP (rPrP) produced in E. coli or mammalian cell

cultures, could be converted into a form that evokes prion disease. An important experiment in this regard was the demonstration that incubation of radioactively labelled PrP^{C} with unlabelled PrP^{Sc} in a cell-free system gave rise to radioactive PrP^{Sc}, as characterized by its physical features (Caughey*, et al.*, 1999; Kocisko*, et al.*, 1994). The *in vitro* reaction also displayed the species- and strain-specific barriers observed *in vivo*, however, no increase in infectivity could be shown.

The invention of the protein misfolding cyclic amplification (PMCA) technique is a significant move ahead in the field of prion research (Saborio, Permanne & Soto, 2001). The PMCA mimics in an accelerated manner, the process of prion propagation. In PMCA, large amounts of PrP^{C} are converted using minute quantities of a brain-derived PrP^{Sc} template. Prion-infected brain homogenate is diluted approximately 1:10,000 with uninfected brain homogenate, incubated and sonicated to fragment aggregates and thereby enhance seeding. Incubation and sonication steps are repeated 20 times in a cyclic manner, hence the name of the technique. The reaction mixture is then diluted 1:10 into uninfected brain homogenate and again subjected to incubation and sonication cycles. After repeated rounds of dilution and amplification, the original infected brain homogenate have been diluted to a point were the original PrP^{Sc} is calculated to have been completely eliminated. Nevertheless, the level of PrP-res remain basically as in the initial 1:10,000 diluted brain homogenate (Castilla *et al.*, 2005). Even more important, infectivity is associated with this *in vitro* amplified PrP-res, as shown by animal bioassays. It is worth noting, however, that incubation time in the animal bioassay is significantly longer than that elicited by the original brain homogenate. This could be due to that the specific infectivity of the newly generated PrP^{Sc} is lower than its "natural" counterpart or because a "strain shift" of the prion occurred. This technique convincingly demonstrate that PrP^{Sc} and infectious agent can be generated *in vitro*; however it does not rule out amplification of other molecules, for example nucleic acids, because it is carried out with brain homogenate and not with purified components. Furthermore, in a recent report Saá and co-workers showed that an optimised version of the PMCA technique is more than 4000-times more sensitive than the animal bioassay (Saa, Castilla & Soto, 2006). The quantity of PrP^{Sc} that this modified PMCA is capable of detecting is approximately equivalent to 26 molecules of PrP, which is similar to recent data on the minimum number of PrP molecules present in a single infectious prion particle (Silveira*, et al.*, 2005). Remarkably, this would indicate that PMCA may be able to detect as little as a single prion.

Another significant step towards final proof of the protein-only hypothesis is the recent reports by Legname and co-workers (Legname*, et al.*, 2004; Legname*, et al.*, 2005). In these experiments a β sheet-rich fibrillar preparation made up by amino-terminally truncated rPrP (comprising residues 89 to 230) was injected intracerebrally into mice overexpressing the same PrP sequence (tg9949 mice). All mice developed neurological disease after about 520 days, as compared with an incubation time of roughly 160 days caused by the "natural" Rocky Mountain Laboratory (RML; also known as Chandler strain (Chandler, 1961)) prion strain. Tg9949 mice injected with only PBS were healthy at 620 days; however, their later fate was not reported. Brain homogenate from the sick tg9949 mice caused

disease after around 260 days when infused into tg9949 mice. Prions in the brains of tg9949 mice inoculated with this recombinant amyloid were designated MoSP1 (mouse synthetic prion strain 1). Significantly, disease could also be transmitted to wild-type mice. MoSP1 in tg9949 were characterised in further detail and were found to differ in several characteristics from mouse-adapted and other prion strains investigated to date (Legname*, et al.*, 2005). Interestingly, passage of MoSP1 through wild-type mice changed the properties of this strain to resemble those of RML prions.

The authors' conclusions of these experiments were that the fibrillar preparation of rPrP contained "synthetic prions," perhaps at low levels, and that these were amplified or underwent a strain shift (or both) in the tg9949-inoculated mice, accounting for the shortened incubation period on further propagation. When MoSP1 was passaged into wild-type mice, the incubation period was decreased from first to second passage. It was suggested that this difference reflects a transmission barrier caused by the difference in PrP sequences between wild-type and Tg9949 mice: wild-type mice express full-length MoPrP, and Tg9949 mice express only the C-terminal 65% of the protein. The change in characteristics of MoSP1 on passage in wild-type mice are reminiscent of the alterations seen when Sc237 prions from Syrian hamsters were passaged in Tg mice expressing chimeric mouse-hamster (MH2M) PrP (Peretz *et al.*, 2002). Furthermore, the incubation times, neuropathologic lesion profiles, and resistance to GdnHCl denaturation indicate that MoSP1 is unlike any prion strain previously described and thus possibly constitute a new strain (Legname*, et al.*, 2005).

It is, however, worth noting that mice which highly overexpress PrP have been shown to spontaneously develop prion-like disease (Chiesa *et al.*, 1998; Westaway *et al.*, 1994b), raising concerns over this mouse model (tg9949). It is thus feasible that tg9949-transgenic mice, which considerably overproduce PrP, would have spontaneously developed prion disease at higher age and that the injection simply accelerated this endogenous process. In fact, a crucial control experiment of injecting brain homogenate from old, noninoculated tg9949 into young tg9949 mice and keep them under observation for the appearance of prion disease have not been reported.

Notwithstanding, the significance of this experiment should not be ignored, to be exact that if prions had not been created in the cell-free system, they must have arisen spontaneously in an uninfected mouse, mimicking the sporadic occurrence of CJD in humans. The interpretation of the Legname experiments are, nevertheless, vital in one respect. If the prions in the tg9949 mice are of endogenous origin then the experiments would not be proof of the protein-only hypothesis. If prions had been generated *in vitro* from recombinant PrP, then the proof would be indisputable, even if amplification required additional components provided by the host, such as RNA (Deleault, Lucassen & Supattapone, 2003).

Present investigations

Aim

The general aims of this study are to investigate the cellular processing of the bovine cellular prion protein and to characterize the molecular features of the Nor98 prion.

Results and discussion

In papers I-III in the appendix, methods used in the present investigations are described in detail and will therefore not be recounted in this section.

Paper I: Proteolytic Cleavage and Shedding of the Bovine Prion Protein in Two Cell Culture Systems

BSE is known to give rise to a zoonotic disease (vCJD) (Collinge, 1999) as a result of bovine PrP^{Sc} crossing the species barrier into humans, which motivated us to perform a detailed study of the cellular processing, turnover and release of bovine PrP^{C}.

Since PrP contains several posttranslational modifications, an eukaryotic expression system is essential for relevant studies of its cellular processing. Furthermore, to be able to study all aspects of the processing in detail, high expression is needed for short pulse-chase radio-labelling analyses. Semliki forest virus (SFV)-vector driven expression of proteins has proven to be an efficient and high-level eukaryotic protein expression system (Liljestrom & Garoff, 1991). The bovine PrP was cloned into the expression vector pSFV1 and used for expression in BHK and N2a cells. The comparison done on the posttranslational processing of bovine PrP in N2a cells with that in BHK cells and the fact that the posttranslational processing of boPrP differs between these cell culture systems is an important new observation. The cleavage pattern of PrP observed in healthy bovine brain, representing the normal *in vivo* cellular processing, contains large amounts of C1 which is also the case in boPrP-expressing BHK cells but not in boPrP-expressing N2a cells. Furthermore, a cleavage in the extreme C-terminus resulted in shedding of boPrP from the cell surface of BHK cells. The high level of expression enabled a short pulse-labelling approach that made it possible to very precisely determine the time-course for the C1 and extreme C-terminal proteolytic clevages. In N2a cells, the majority of boPrP was subjected to a more complete degradation process, and only trace amounts of full length boPrP was shed into cell culture medium in a process which also indicated a release by proteolytic cleavage.

The results in this paper revealed that shedding of PrP into the extracellular medium, represent an important step in the normal posttranslational processing of boPrP. The observation that this shedding is protease-mediated is interesting as many GPI-achored proteins exert their functional role when proteolytically

released from its GPI-anchor (Kahl *et al.*, 2000; Tsuji, Kaji & Nagasawa, 1994). Thus, this observation can be important with regard to functional aspects of PrP^C.

Paper II: Exosome- and Protease-mediated Shedding of the Bovine Prion Protein is Unaffected by Deletion of the C1 Cleavage Site

The constitutive proteolytic shedding is an important posttranslational processing event in the biosynthesis of PrP. Its importance for functional aspects of PrP^C and its role in prion pathogenesis is unknown. This motivated us to study the cell surface shedding in detail. Interestingly, during the course of these studies a mechanism of release distinct from the protease-mediated was discovered. Further analyses identified this mechanism as being exosome-mediated. In addition, the importance of the C1 cleavage for these different mechanisms of release was investigated.

In order to investigate the influence of the C1 cleavage on the shedding of boPrP from the cell, a deletion mutant of the cleavage site was studied. Although the cleavage was partially hindered, the export of PrP to the medium was unaffected. Two distinct released PrP populations could be found in the cell medium. The major fraction was released by proteolysis near the GPI anchor. The other, minor fraction, was released in association with exosomes as separated out by differential ultracentrifugation and further characterized by Western immunoblotting with exosome-specific markers, phospholipase assays, and electron microscopic analysis. Less then 1% of the released boPrP was exported in association with exosomes. When the protease-mediated shedding was inhibited by the use of a zinc metalloprotease inhibitor, exosome-mediated shedding of boPrP was not affected, suggesting separate pathways and a constitutive shedding of exosome-associated boPrP. Indications of a low level of phospholipase-mediated shedding was observed, probably due to phospholipase activities in the fetal calf serum present in the cell culture medium.

Taken together, the results presented in this study indicate that a deletion of the C1 cleavage site inhibits, albeit only partially, the cleavage of full length boPrP into C1. However, the slowed rate of C1 cleavage results in profound changes in the composition of boPrP shed from the cell into the extracellular medium. The finding that boPrP in addition to a protease-mediated release also is released in association with exosomes, provide important information in relation to functional aspects of PrP^C and possible roles in pathogenesis.

Paper III: Characterization of Proteinase K-resistant N- and C-terminally Truncated PrP in Nor98 Atypical Scrapie

In this paper, a newly discovered prion disease of sheep, Nor98, was characterized with regard to its molecular properties. Some features of the PK-resistant PrP fragments in Nor98 resembled those of PrP fragments associated with the human prion disease Gerstmann-Sträussler-Scheinker syndrome.

An increasing number of scrapie cases with atypical characteristics, designated Nor98, have recently been recognized in many countries in Europe. In this paper,

the proteinase K (PK)-resistant prion protein (PrP) fragments from two Swedish cases of Nor98 atypical scrapie was characterized. The prominent, fast-migrating band in the distinct Nor98 Western immunoblot electrophoretic profile was determined to be of 7 kDa in size and was accordingly designated Nor98-PrP7. The antigenic composition of Nor98-PrP7, as assayed by a panel of anti-PrP antibodies, revealed that this fragment comprised a mid-region of PrP from around aa 85 to 148. N- and C-terminally truncated fragments spanning the mid-region of PrP have previously only been observed in the genetic prion disorder Gerstmann-Sträussler-Scheinker syndrome. It is shown in this paper that the long-term PK resistance of Nor98-PrP7 is reduced compared with that of PrP-res in classical scrapie. Enzymatic deglycosylation did not change the distinct electrophoretic profile of Nor98-PrP7. 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. Antigenic determination and the size of 14 kDa suggested a fragment spanning approximately aa 120-233. The existence of two PK-resistant PrP fragments, Nor98-PrP7 and PrP-CTF14, that share an overlapping region suggests that at least two distinct PrP conformers with different PK-resistant cores are present in brain extracts from Nor98-affected sheep. The structural gene of PrP in three Nor98-affected sheep was analysed, but no mutations were found that could be correlated to the aberrant PK-resistant profile observed.

In conclusion, this study shows that the PK-resistant PrP fragments present in Nor98-affected sheep display striking similarities to those found in individuals affected by the human prion disorder Gerstmann-Sträussler-Scheinker syndrome (GSS). Interestingly, GSS is always associated with aa substitutions in the PrP. Differently, no changes in the PrP of Nor98-affected sheep have been found. The findings presented in this paper together with observations of a distinct epidemiology, suggest that Nor98 could be the result of an age-related spontaneous conversion of PrP^{C} into PrP^{Nor98} similar to that proposed for sCJD and sFI.

Concluding remarks and future perspectives

In order to elucidate the underlying molecular basis of prion diseases, continued thorough studies of the normal cellular prion protein must be conducted. If the function(s) of PrP^C is clarified, most probably it will result in new approaches for therapeutic interventions and new targets for pharmacological treatment strategies.

The present investigations reveal that the posttranslational processing of boPrP differ significantly between cell lines, emphasizing the importance of knowledge of the cell culture model used for studies of PrP. The findings further underscores the significance of differences in specific protease activities in different cell lines. The knowledge that cleavage into C1 occurs *in vivo* in all species studied to date is crucial for the choice of cell culture models and antibodies for detection of PrP and fragments thereof. Against the background of this, these studies demonstrate conclusively that deglycosylation is a prerequisite for any type of studies of the cell biology of PrP^C and PrP^{Sc} and that it must be used in combination with different PrP-antibodies to detect full length as well as both N-terminal and C-terminal fragments of PrP. Of particular note is mAb 3F4, the epitope of which covers the C1 cleavage site and is thus destroyed upon posttranslational cleavage of full length PrP into C1. This epitope is naturally present in human and hamster PrP, and mAb 3F4 has an absolute requirement for the sequence $M_{109}K_{110}H_{111}M_{112}$ (huPrP numbering) (Kascsak *et al.*, 1987). As this epitope is not present in mouse PrP (moPrP), insertion of a "3F4-tag" of this sort into moPrPs expressed exogenously by transfection is frequently used to be able to separate it from endogenous moPrP present in cell lines of murine origin. A better choice in this regard is to insert the epitope of mAb L42, which has a requirement for W at position 145 (huPrP numbering) (Vorberg *et al.*, 1999). In addition to identification of exogenously expressed moPrP this "L42-tag" enables detection of both full length and C1 PrP.

The mouse neuroblastoma cell line N2a is the cell line of choice for studies of mouse-adapted scrapie and also for studies of PrP^C from various species. However, in light of our findings that boPrP is efficiently degraded and does not undergo the C1 cleavage in N2a cells, caution should be taken when performing studies of the cellular processing of PrPs in this cell line.

Interesting future investigations include creating mutants of PrP that affects the proteolytic shedding and the C1 cleavage and study the role of these processing events for PrP^C function and prion propagation. The separation of boPrP released by the two distinct mechanisms into a pellet and a supernantant fraction, respectively, could prove to be a useful tool for characterisation of PrP populations associated with infectivity. Moreover, since exosomes from infected cells have been shown to be infectious, it would be interesting to investigate if also the PrP population in the 100,000 x *g* pellet from medium conditioned with prion-infected cells, contain any infectivity. If that is not the case, a therapeutic approach to treat prion infection could be to increase the proteolytic shedding in order to reduce the amount of PrP^C available for subsequent conversion into PrP^{Sc} and release from

the cell via exosomes. In this regard, identification of the proteases responsible for the proteolytic shedding could be of great interest. Construction of mice deficient for proteases involved in C1 cleavage and/or shedding could provide important insights to the function of PrP^C and the role of these processing events in prion pathogenesis.

The characterization of the Nor98 prion presented in this thesis reveal striking similarities with the GSS prion as discussed above. As both Nor98 and GSS prions display relatively low PK-resistance it would be interesting to investigate if anomalous association with membranes in the cell could explain the unusual properties rather than a typical aggregation process, as transmembrane forms of PrP have been identified in models of GSS (DeArmond & Prusiner, 2003; Harris, 2003; Hegde *et al.*, 1998; Hegde *et al.*, 1999; Kim, Rahbar & Hegde, 2001; Stewart & Harris, 2001). In this regard it would be exceedingly useful to set up a cell culture model infected with Nor98 to study the molecular basis of this disease.

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