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Cellular Prion Proteins in Human Platelets Show a Phenotype Different to Those in Brain Tissues

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ABSTRACT

Prion diseases are characterized by high accumulation of infectious prion proteins (PrP^{Sc}) in brains. PrP^{Sc} are propagated by the conversion of host-encoded cellular prion proteins (PrP^{C}) which are essential for developing the disease but are heterogeneously expressed in brains. The disease can be transmitted to humans and animals through blood and blood products, however, little attention has been given to molecular characterization of PrP^{C} in blood cells. In this presented study, we characterized phenotypically PrP^{C} of platelets (plt) and characterized the proteins regarding their glycobanding profiles by quantitative immunoblotting using a panel of monoclonal antibodies. The glycosylation patterns of plt and brain PrP^{C} were compared using the ratios of di-, mono-, and non-glycosylated prions. The detergent solubility of plt and brain PrP^{C} was also analyzed. The distinct banding patterns and detergent solubility of plt PrP^{C} differed clearly from the glycosylation profiles and solubility characteristics of brain PrP^{C}. Plt PrP^{C} exhibited single or only few prion protein types, whereas brain PrP^{C} showed more extensive banding patterns and lower detergent solubility. Plt PrP^{C} are post-translational modified differently from PrP^{C} in brain. These findings suggest other or less physiological functions of plt PrP^{C} than in brain. J. Cell. Biochem. 112: 954–962, 2011.

KEY WORDS: PLATELET; PRION PROTEIN; CREUTZFELDT–JAKOB DISEASE; PHENOTYPE

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that involve proteins called prions. Human prion diseases comprise sporadic and genetic forms known as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), Fatal Familial Insomnia (FFI), and Kuru. The prion diseases transmitted by infection include variant CJD (vCJD), which has been linked to the prion disease of cattle known as the bovine spongiform encephalopathy (BSE) [Collinge et al., 1996]. Human blood has emerged as a potential route of prion disease infection. Four cases of transmission of vCJD through blood transfusions have recently been reported [Llewelyn et al., 2004; Peden et al., 2004]. This caused serious concern regarding the danger and risk of TSE transmission by blood transfusion involving unrecognised, asymptomatic but infectious donors in the donor population.

TSE infectivity has also been demonstrated in a variety of peripheral tissues, and body fluids such as blood may also contain the infectious agent [Brown et al., 1999; Taylor et al., 1999; Houston et al., 2000; Holada et al., 2002; Cervenakova et al., 2003]. It has been shown that the BSE agent can be transmitted to sheep by transfusion of whole blood [Houston et al., 2008]. These findings indicate the need to continue developing preventive measures against the transmission of prion diseases. In the case of blood products, this means the development of methods for prion reduction and the development of highly sensitive screening assays to identify blood donors capable of transmitting the disease [Holada et al., 2007].

Prion diseases are characterized by the occurrence of a pathological prion protein termed PrP^{Sc} which is believed to be the major if not sole pathological component of the disease [Prusiner 1998]. PrP^{Sc} is the result from the post-translational conversion of the host-encoded cellular prion protein (PrP^{C}), whose presence is essential for the propagation of PrP^{Sc} and development of the disease. PrP^{C} is predominantly found on peripheral neurons and in the central nervous system [Kretzschmar et al., 1986; Bendheim et al., 1992], but it is also present on the surface of human B- and T-lymphocytes, on monocytes and macrophages, denditric cells,

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natural killer cells and on CD34-positive hematopoetic stem cells derived from bone marrow [Cashman et al., 1990; Dodelet and Cashman, 1998; Durig et al., 2000; Holada and Vostal, 2000]. After its expression, PrP^C is modified post-translationally at the amino- and carboxyl-terminal ends. The protein structure bears one disulphide bond between cystine residues and two asparagine-linked glycosylation sites which give rise to the occurrence of di-, mono-, and non-glycosylated forms of PrP^C. The protein is attached to the outer cell membrane through a glycophosphatidyl-inositol-(GPI-) membrane anchor. Little is known about the physiological function of PrP^C. The protein has been associated with enzymatic and signaling functions, metal ion binding and transport [Lasmézas, 2003; Jouvin-Marche et al., 2006; Aguzzi et al., 2008].

PrP synthesis in the blood cells of mammals is highly variable and in human blood most of the PrP^C is located in the platelet (plt) and plasma fractions where it is present in high amounts [MacGregor et al., 1999; Fagge et al., 2005; Jones et al., 2005]. In resting plt, the protein is mainly associated with membranes and localized in the α-granules [Holada et al., 2006]. On activation the surface expression of PrP^C is elevated and the protein, in part, is released from plt via shedding of microvesicles and exosomes [Holada et al., 2006; Robertson et al., 2006]. Prions in brain and tissues are heterogeneous with regard to their expression and protein types reflecting differences in the ratios of the signal intensities of the di-, mono-, and non-glycosylated isoforms. Two different glycosylation profiles have been detected in human brain, one located predominantly in the cortex with high-intensity signals associated with the di-glycosylated isoform and a second, where the non-glycosylated isoform is dominant, found in the pons region [Kuczius et al., 2007b].

Up to now, little attention has been given to the molecular analysis of PrP in blood cells. In this study, we systematically analyzed PrP^C from plt using quantitative immunoblot assays and compared the biochemical characteristics with PrP^C obtained from brain tissues. Various antibodies were used which recognize PrP within its octapeptide region, intermediate sequences, core protein, and carboxyl-terminal amino acids. An analysis of the PrP^C glycoforms was performed by quantification of the signal intensities of the di-, mono-, and non-glycosylated PrP^C as well as the amino truncated isoforms of PrP^C. Our results show PrP^C glycosylation patterns in plt which clearly differ from brain PrP^C, since they show abundant signal intensities of the diglycosylated proteins independent of the antibody used for detection. Thus, PrP^C expression seems to be modified in blood plt in a way different to that in brain.

MATERIALS AND METHOD

ANTIBODIES FOR THE DETECTION OF PRION PROTEINS (PrP^C)

Monoclonal anti-PrP antibodies (mabs) SAF34, SAF60, and SAF70 were previously prepared by immunizing PrP knock-out mice. The immunogens were formic acid-denatured scrapie-associated fibrils (SAF) which were obtained from hamster brains infected with strain 263K [Demart et al., 1999]. Mabs Pri308 and Pri917 were obtained using a synthetic peptide of human PrP as immunogen [Morel et al., 2004], and mabs 8G8 and 12F10 were raised against recombinant human PrP [Krasemann et al., 1996]. The antibodies were applied as ascitis derived from mice and in this study were obtained from one single lot. Purified mab 3F4 (Signet) was reactive to amino acid residues 109–112 of prion protein [Kascsak et al., 1987]. The epitopes recognized by the various antibodies are listed in Table I. The brain marker neuron-specific enolase (NSE), which was used as a control in the blots, was detected by the monoclonal mouse anti-NSE antibody which was supplied as purified IgG (Dianova, Hamburg, Germany).

ISOLATION AND PREPARATION OF HUMAN PLATELETS (PLT)

Platelets were isolated from 10 ml ethylene diamine tetra-acetic acid (EDTA)-anticoagulated blood samples obtained from 10 healthy volunteer blood donors after obtaining their informed consent. Plt-rich plasma was prepared by centrifugation at $240 \times g$ for 10 min, carefully removed to prevent contamination with leukocytes and was analyzed individually and pooled. Plt were diluted with isotonic saline containing 0.5% (w/v) EDTA and spun down at $1{,}500 \times g$ for 10 min. After washing three times with Dulbecco-PBS (pH 6.5; Invitrogen, Karlsruhe, Germany), the plt concentration was adjusted to $4 \times 10^6/\mu l$ using a Sysmex K1000 counter (Sysmex, Norderstedt, Germany). For typing experiments, plt were lysed in Tris-buffered saline (TBS; 20 mM Tris/HCl and 150 mM NaCl) containing 0.5% SDS, 0.5% igepal-CA630 and 320 mM sucrose. Plt lysates were stored in aliquots at −70°C for up to 6 months. For experimental analysis, individual plt preparations were investigated for individual differences and a pooled suspension based on single donors was used as standard suspension.

TABLE I. PrP-Specific Monoclonal Anti-PrP Antibodies Used

Monoclonal antibody	Isotype	Recognition region	Linear epitope	Immunogen
SAF34	IgG2a	Octapeptide region	59–89	Hamster scrapie
8G8	IgG2a	Intermediary region	97–102	Human peptide
3F4	IgG2a	Intermediary region	109–112	Hamster scrapie
Pri308	IgG1	Intermediary region	111–118	Synthetic peptide
12F10	IgG2a	Core protein region	144–152	Recombinant human PrP
SAF60	IgG2b	Core protein region	157–161	Hamster scrapie
SAF70	IgG2b	Core protein region	156–162	Hamster scrapie
Pri917	IgG1	Carboxy-terminal region	216–221	Synthetic peptide

BRAIN TISSUE PREPARATIONS

Samples of brain tissues were obtained from six human subjects who were free of prion diseases and other neuropathological diseases. The brain tissues were obtained from the Institute of Neuropathology Brain Bank at the University Hospital Münster. Consent for autopsy and scientific examination was obtained from the legal representatives (Ethics Committee of the Westphalia Chamber of Physicians and the Faculty of Medicine, University of Münster) for all subjects in accordance with the local institutional review board requirements. Brain tissues derived from pooled tissues of several but not clearly specified brain regions which mostly composed of cortex and cerebellum and consisted of medulla oblongata, nucleus lentiformis, thalamus, hippocampus, and pons to lower fractions. Tissues were suspended in nine volumes of TBS (pH 7.4) and 10% (w/v) homogenates were prepared. The experiments were carried out with one charge of a pooled homogenate obtaining standardized conditions. For typing assays, an aliquot of the pooled tissues were lysed in homogenate buffer consisting of TBS with SDS and igepal-CA630 at concentrations of 0.5% (w/v) each and 320 mM sucrose [Kuczius et al., 2007a]. Tissues were minced using pestles in glass tubes and homogenizers driven by an electric motor followed by ultrasonification for 30–60 s as previously described [Kuczius et al., 2007a]. The cell debris was removed by centrifugation at $900 \times g$ for 5 min and the supernatants were stored at -20°C until use.

ENZYMATIC TREATMENT OF PROTEIN SAMPLES

Enzymatic deglycosylation was carried out as described previously [Kuczius et al., 2007b]. Briefly, SDS was applied to the samples to give a final concentration of 1.5% (w/v). For pre-denaturation, the protein samples were diluted 2.5-fold in TBS containing 10 mM EDTA, 1% (w/v) igepal-C630, and 1.5% (v/v) mercaptoethanol and heated at 99°C for 10 min. The proteins were deglycosylated with 1 unit *N*-glycosidase F (PNGase F; Roche, Mannheim, Germany) at 37°C for 16 h. The reaction was terminated by addition of SDS-loading buffer to the samples.

DETERGENT SOLUBILITY OF PrP^C

Differential solubility experiments were carried out with protein samples suspended in TBS buffer. Plt and brain tissue suspensions were pre-treated with the detergents to determine the solubility characteristics. For analyzing the solubility of PrP^C from plt and brains, samples were incubated with anionic detergents such as *N*-lauroylsarcosine (sarcosyl) and sodium dodecyl sulfate (SDS) each in a concentration of 1%. *N*-octyl-β-D-glucopyranoside (OGP) and Triton X100 (triton), representing the non-ionic detergents, were applied in concentrations of 2% and 1%, respectively. CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid), a zwitterionic detergent, which is especially useful for the purifcation of membrane proteins, was used at a concentration of 2%. The detergents were added to protein suspensions and the mixture incubated at room temperature and 4°C for 30 min and 16 h, respectively. To prepare fractions of low and highly soluble prion proteins, the suspensions were centrifuged in Eppendorf tubes at $16{,}000 \times g$ for 10 min providing supernatants (S) and pellets (P). Pellets were washed and resuspended in TBS comprising the primary detergent.

IMMUNOBLOT ANALYSIS

Samples were resuspended in SDS-loading buffer and denatured at 99°C for 5 min. Proteins were analyzed using sodium dodecyl sulphate polyacrylamide (13%) gel electrophoresis (SDS–PAGE) in a mini slab gel apparatus (Bio-Rad, Munich, Germany). After electrotransfer onto polyvinylidene difluoride membranes (Immobilon-P; Roth, Karlsruhe, Germany) using a semi-dry blotting system (Roth), membranes were blocked in TBS with 0.05% Tween 20 (TBST) and 1% (w/v) non-fat dry milk powder for 1 h at room temperature followed by incubation with primary antibodies at room temperature overnight. After washing with TBST, the membranes were incubated for 2 h at room temperature with peroxidase-conjugated goat anti-mouse IgG antibodies (Dianova). Specific protein signals were visualized using a chemiluminescence enhancement kit (Pierce, Bonn, Germany).

QUANTIFICATION AND TYPING OF PRION PROTEINS

Immunoblots were scanned on a chemiluminescence photo-imager (Bio-Rad) and the relative amounts of the bands of interest were obtained by computerized integration of peaks representing the bands using the QUANTITY ONE software (Bio-Rad). The combined PrP^C signal with one sample, comprising the sum of the signals for di-, mono-, and non-glycosylated isoforms, was defined as 100% and the percentage of each band was calculated. Protein profiles were analyzed by calculation of arithmetic means of the samples after immunoblotting. Variations in repeated SDS–PAGE runs are expressed as errors of means (SE). For statistics, mean percentage data of di-, mono-, and non-glycosylated isoforms were normalized by calculation of the Shannon diversity index, and statistical analysis was performed using the Mann–Whitney *U*-test and was conducted using the SPSS software. P values of < 0.05 were considered to be significant.

RESULTS

DETECTION OF PRION PROTEIN SIGNALS IN PLATELETS

Cellular prion proteins of plt isolated from human blood were analyzed by immunoblotting with anti-PrP monoclonal antibodies. Plt PrP^C revealed three characteristic protein bands detected with all antibodies which are used in this study and which bind to epitopes at the amino-terminal, the intermediary, the core, and the carboxyl-terminal protein regions (Fig. 1). The band with the lowest molecular mass represents the non-glycosylated isoform, whereas the two protein bands with higher molecular weights are mono- and diglycosylated isoforms. For quantification, the densitometrical measurement of the protein signal intensities was carried out within the linear range of light emission which was determined by analysis of serially diluted protein suspensions (Fig. 2). The linear range was defined as 1×10^7 to 5×10^7 cells/μl, because PrP^C was readily detectable in this range and the glycoprofiles showed similar quantitative patterns for the three isoforms. Individual PrP^C plt preparations exhibited identical patterns and had the same profiles as pooled fractions (not shown).

PROTEIN TYPING OF PLATELET AND BRAIN PrP^C

The banding patterns were analyzed by immunoblotting using several monoclonal anti-PrP antibodies which recognize different

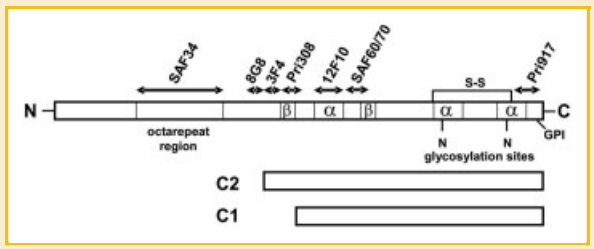

Fig. 1. N, amino-terminal region; C, carboxyl-terminal region; GPI, glycophosphatidyl-inositol anchor.

epitopes within the protein sequence (Table I). Each PrP^C protein profile was quantified by chemiluminescence. For determination of the banding profiles, the combined signals of the three PrP^C bands were defined as 100% and corresponding ratios of signal intensity for each band were calculated (Fig. 3). All three protein bands were detected with each of the used antibodies in this assay, independently on the antibody recognition sites. As no divergent plt-derived PrP^C isoform patterns of the individual blood donors were observed (not shown) a pooled protein suspension was used for standardized analyses. The diglycosylated isoforms of plt PrP^C predominated when antibodies SAF34, 8G8, 3F4, and Pri308 were used which bind to the octapeptide region and up to amino acid 120 defined as the intermediary region. The intensities of the diglycosylated bands constitute 86–93% corresponding to the total PrP^C signal. The signals of the monoglycosylated isoforms were low (9–14%) and the non-glycosylated proteins were hardly detectable. This uniform banding profile differed considerably and significantly from PrP^C derived from brain tissues. The diglycosylated isoforms in human brain gave consistent signals but exhibited markedly lower intensities (54–57%). In the following investigation, the proteins were typed using antibodies such as mabs 12F10, SAF60, and SAF70 which recognize the core protein region. Significant differences were shown with antibodies 12F10 and SAF70. The diglycosylated isoforms of plt PrP^C changed to a lower intensity (39–67%) in favor to a protein band with the molecular mass at the non-glycosylated protein (29–53%). The high signal intensity of the lower band may result from an overlay of full-length non-glycosylated proteins and glycosylated but truncated isoforms. The monoglycosylated band in plt exhibited low signals (<9%). These glycoprofiles clearly differed from PrP^C patterns of brain tissues. With brain, PrP^C was always dominant in the band with a kDa size corresponding to that of the non-glycosylated proteins (73–87%) which also consisted of an overlap of full-length and truncated isoforms. When using mab Pri917, which binds to the carboxyl-terminal PrP sequence, the non-glycosylated protein of plt PrP^C exhibited the highest signal intensities (73%). This result is in contrast to brain PrP^C where diglycosylated isoforms were dominant (54%). Reproducibility of the protein banding patterns was evaluated with samples of individuals and with pooled samples derived from the same sources (not shown). The differentially expressed banding patterns, attributed to antibody recognition sites within the amino- or carboxyl-terminal PrP sequences, have been described earlier [Kuczius et al., 2007a]. Dominant diglycosylated proteins were detected with antibodies binding to the amino-terminal and intermediary regions and high signal intensities were determined corresponding to a protein band at the size of the non-glycosylated full-length prion protein consisting of non-glycosylated full-length isoforms and glycosylated truncated fragments when using antibodies which recognize epitopes at the carboxyl-terminal region. Different banding patterns of PrP^C were also observed in various brain regions when using amino-terminal binding antibodies for detection [Kuczius et al., 2007b]. PrP^C in the cortex, nucleus lentiformis, thalamus, hippocampus, and cerebellum showed high signal intensities with the diglycosylated isoforms. These patterns clearly differed from the types derived from pons and medulla oblongata which displayed high staining of the non-glycosylated isoform. According to the results described here it is evident that the PrP^C glycoprotein profiles of plt are different from the PrP^C patterns identified in brain tissues.

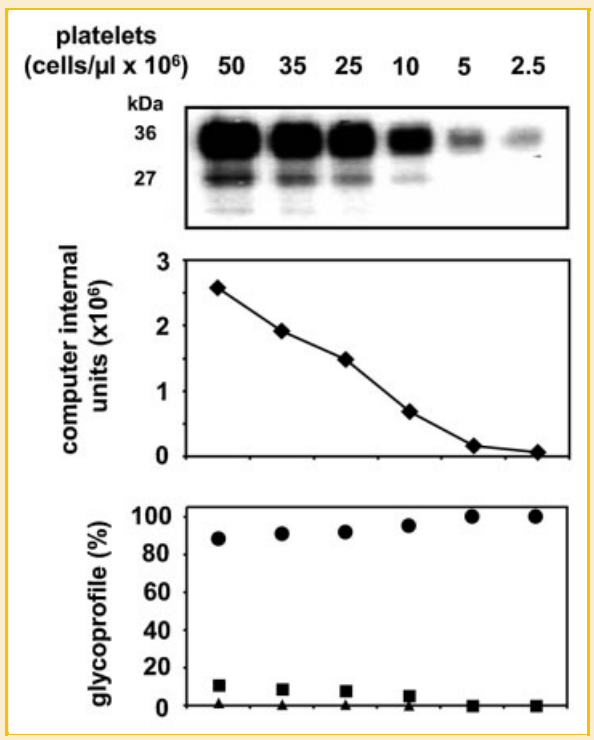

Fig. 2. Immunoblot analysis and determination of the linear range of PrP^C signals. Immunological detection of serial diluted PrP^C suspensions derived from platelets using the antibody SAF34, which recognizes the octapeptide region. PrP proteins were quantified densitometrically for determination of the linear range of reaction. The total PrP signals consisting of non- and glycosylated isoforms are given as computer internal units (upper graph). PrP^C were typed regarding the ratios of the measured signal intensities of the di- (circle), mono- (square), and non-glycosylated (triangle) isoforms.

AMINO-TERMINAL TRUNCATION OF PLATELET PRPC

High signal intensities at the size of non-glycosylated PrP^C were observed using antibodies recognizing sequences within the core protein. This signal dominance may result from a protein overlay and/or from truncation, which occurs naturally in brain. PrP^C will be modified endogenously by proteolysis forming amino-terminal

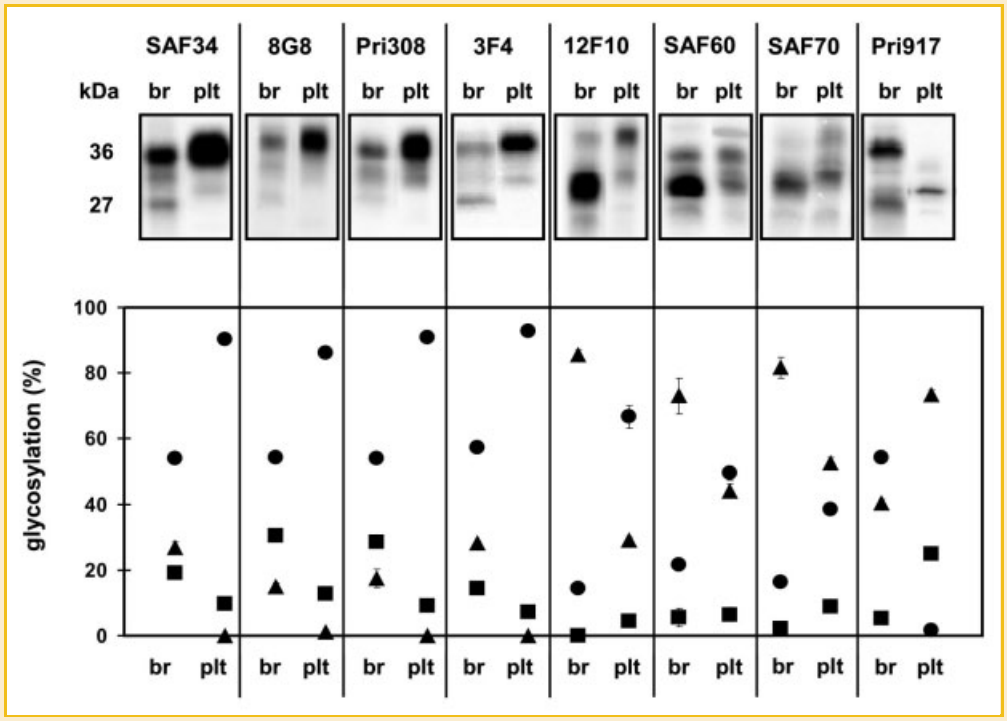

Fig. 3. Protein typing of platelet and brain PrP^C. For glycotyping PrP^C proteins derived from platelets (plt) and brain homogenates (br), the ratios of the di- (circle), mono- (square), and non-glycosylated (triangle) isoforms were measured as percentages of the total signals and calculated as arithmetical means (±SE of the mean) of separated gel runs. The number of gel runs for the analyses are given for each antibody. Calculation of the protein profiles of brain and platelet PrP^C of 14 and 26 gel runs, respectively, using antibody SAF34 gave SE values of 1.5 for the di-glycosylated isoforms, 0.5 for the mono- and 1.7 for the non-glycosylated brain PrP^C and 0.5 for the di-glycosylated platelet PrP^C isoforms, 0.5 for the mono- and 0.1 for the non-glycosylated protein, respectively; 8 and 19 runs using antibody 8G8 (SE 1.5; 1.3; 1.0 for brain; 1.6; 1.4; 0.5 for platelets); 10 and 15 gels for antibody Pri308 (SE 1.1; 2.1; 2.7 for brain; 0.8; 0.8; 0.5 for platelets); four and seven gels for antibody 3F4 (SE 1.4; 1.0; 0.5 for brain; 0.8; 0.8; 0.2 for platelets); 14 and 16 gels for antibody 12F10 (SE 1.4; 0; 1.4 for brain; 1.6; 1.3; 0.9 for platelets); 7 and 13 gels for antibody SAF60 (SE 3.4; 2.8; 5.5 for brain; 0.9; 2.1; 2.1 for platelets); 10 and 30 gels for antibody SAF70 (SE 2.0; 1.3; 3.1 for brain; 1.0; 1.6; 1.7 for platelets); 9 and 24 gels for antibody Pri917 (SE 1.0; 0.4; 1.1 for brain; 0.4; 1.4; 1.4 for platelets). Statistical analysis of PrP^C derived from brain and derived from platelets gave significant results for all tested antibodies ($P < 0.05$) except data obtained from antibody SAF60.

truncated proteins referred to as C1 and C2 [Jiménez-Huete et al., 1998]. The non-glycosylated C1 fragments run at 18–19 kDa, whereas the C2 proteins migrate at approximately 21–22 kDa. In this study, plt PrP^C was examined for evidence of truncation, that is, for spots similar to those found with PrP^C in brain tissues. As expected, the amino-terminal binding antibody SAF34 bound full-length PrP only after enzymatic deglycosylation (Fig. 4), whereas mab Pri308 also detected the C2 fragment. The core protein-binding antibody 12F10 additionally recognized the C1 fragment at 18–19 kDa. As the cleavage site forming the C1 protein is at amino acid residues 110–112, this fragment is not detectable by Pri308 as expected but is detectable with 12F10. At the size of the non-glycosylated isoform we identified three bands which are similar in the molecular mass. These were not seen with brain tissue so that it may be related to the preparation.

Our results indicate that PrP^C from plt has recognition sites for the formation of the amino-terminal truncated isoforms C1 and C2 identical to brain PrP^C.

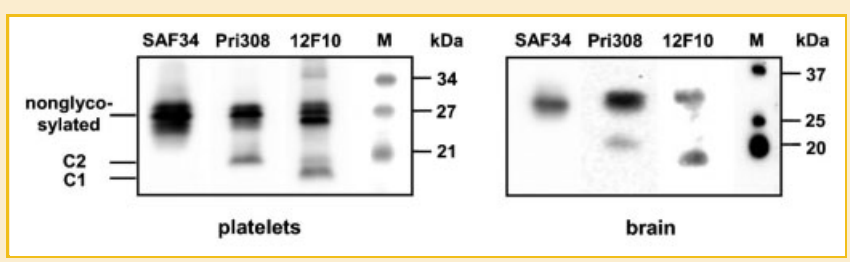

Fig. 4. Immunoblot analysis of deglycosylated PrP^C. Platelet suspensions were treated with PNGaseF for deglycosylation of proteins. After immunoblotting the membranes were probed with antibody SAF34, Pri308, and 12F10, respectively. The protein bands were detected as full-length non-glycosylated isoform (~27 kDa) and the truncated isoforms C2 and C1 at ~21–22 and 18 kDa, respectively.

SOLUBILITIES OF PLATELET AND BRAIN PRPC

Since considerably higher signal intensities were detected for the diglycosylated isoforms in plt, the plt-PrP^C seems to be post-translational modified differently from that in brain. However, PrP^C profiles are mainly the result of an overlay of various protein isoforms [Kuczius et al., 2009]. In order to identify overlaying proteins, the differential solubility of overlapping PrP^C isoforms were analyzed (Fig. 5). The application of detergents to the protein samples resulted in different protein solubilities and enabled the identification of highly soluble and slightly soluble protein forms. After addition of the detergents it was possible to separate PrP^C into high- and low-soluble protein fractions by centrifugation. High intensities of highly soluble plt PrP^C were found on immunoblots probed with SAF70. The addition of the zwittergent CHAPS and the polar detergents sarcosyl and SDS resulted in less higher solubility (95–96%) than with the non-ionic detergents triton and OGP (84%). This high protein solubility was not found for PrP^C derived from brain tissues. Highly soluble protein fractions were identified in the range of 68–79% with SDS, sarcosyl and OGP, whereas not more than 61% were highly soluble in the solution with CHAPS. A high portion of PrP^C protein (69%) had a low-detergent solubility using the detergent Triton-X100. Our data indicate a considerable increased solubility of PrP^C proteins derived from plt in comparison to PrP^C obtained from brain tissues.

DISCUSSION

TSE diseases are characterized by the accumulation of pathological prions (PrP^{Sc}) with their specific variations. The formation of PrP^{Sc} results from protein conversion of the host-encoded PrP^C and therefore the expression of PrP^C in tissues is a prerequisite for the formation of PrP^{Sc}. Many PrP^C subtypes have been identified with multifaceted glycosylation profiles and distributions in several brain regions [Sales et al., 1998; Beringue et al., 2003; Kuczius et al., 2007b]. PrP^C is differentially expressed and distributed in blood cells which are shown by flow-cytometric methods [Holada et al., 2007]. In human blood, more than 80% of the total cell-associated PrP^C is located in plt [MacGregor et al., 1999], whereas the highest levels in blood cells were found in subpopulations of T-lymphocytes [Choi et al., 2009]. However, little is known about the banding profiles and the protein types in blood cells. In the present study, we systematically analyzed the PrP^C glycoform profiles of plt PrP^C using several antibodies recognizing the octapeptide, intermediate,

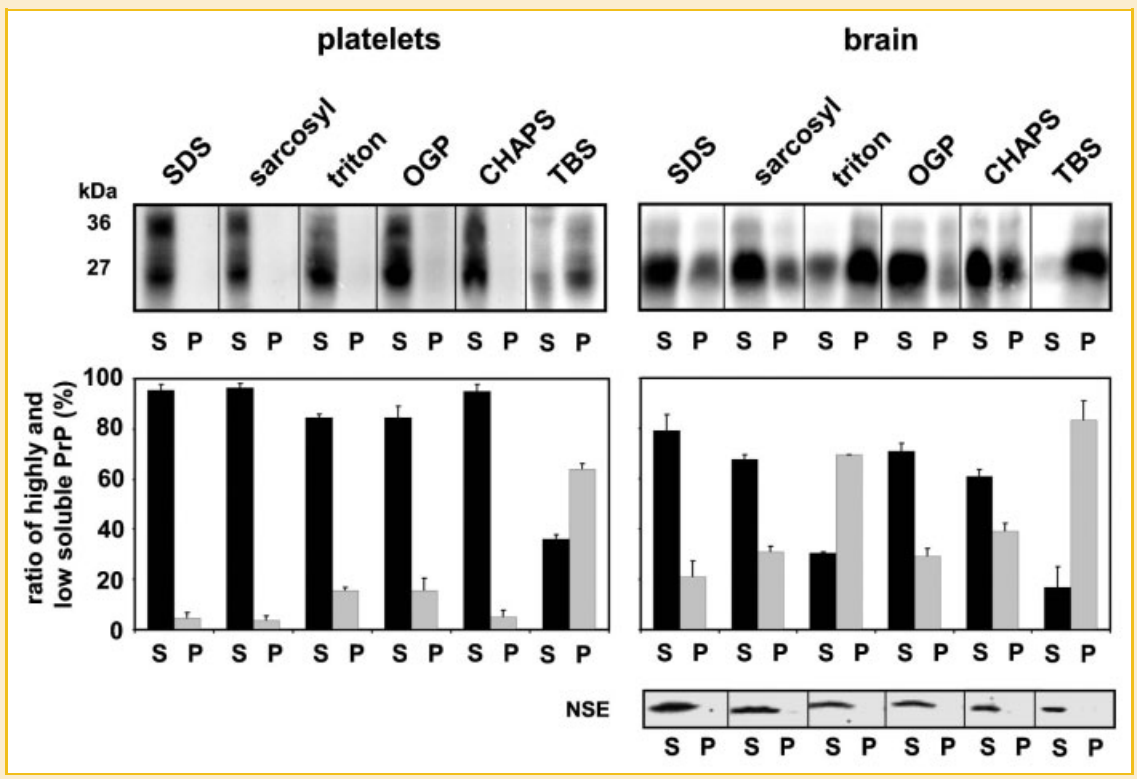

Fig. 5. Solubility of human prions derived from platelets and brain. Proteins of platelets (10^7 cells/μl) and brain homogenates (10%) were suspended in TBS and applied with the following detergents: SDS (1%), sarcosyl (1%), Triton X100 (1%), OGP (2%), CHAPS (2%), and TBS buffer. Samples were divided into highly soluble and low soluble protein fractions by centrifugation. Pelleted proteins were washed and resuspended in TBS with the applied detergent in the original volume. Proteins were immunoblotted, and prions with high or low solubility were detected in the supernatants (S) and the pellets (P), respectively. PrP was specifically detected using mab SAF70. PrP signals were analyzed densitometrically and signals were quantified using the Quantity One software. The percentages of prions with high and low solubility from at least three independent gel runs are given as arithmetical means (±SE). The neuron-specific enolase (NSE), which is an indicator for brain tissues and served as a control protein, was found only in the supernatants.

core protein, and carboxyl-terminal regions in the PrP sequence and compared them with protein profiles of brain. PrP^C proteins with different profiles may play a role in specific physiological functions as signalling and metal bindings which remain to be determined. Thus, the nature of plt PrP^C has to be analyzed. Furthermore, PrP^C may act as a substrate for the assembly of PrP^{Sc} according to the protein–protein interaction theory so that quantity and quality of PrP^C types can play an important role in formation of specific PrP^{Sc} types. The molecular glycoprotein typing of PrP^C presented in this study is based on differences in the relative signal intensities of the di-, mono-, and non-glycosylated isoforms which were sensitively detected with the antibodies used here.

A high-signal intensity of the diglycosylated isoforms of plt PrP^C predominated when using antibodies recognizing the octapeptide and intermediate regions. This dominance diminished step by step and changed to an increase of the protein intensity at the kDa size of the non-glycosylated isoform using antibodies recognizing epitopes of the core protein. In contrast, brain PrP^C exhibited two different but distinct profiles under these conditions. The diglycosylated proteins were abundant when detecting prions with antibodies binding to the octapeptide and intermediate regions, whereas the intensities of the non-glycosylated proteins dominated with antibodies binding to the core protein and the carboxyl-terminal sequence. The difference between the PrP glycosylation profiles in plt and brains indicates a different post-translational modification of PrP^C in plt compared to brain PrP^C. However, antibody reactivity does not always correlate with the ability to detect PrP^C isoforms from different sources (Barclay et al., 2002). The structure may vary with tissue and cell specific transcripts, post-translational modifications and topology, and subtle alternations in the protein conformation and glycosylation may change the accessibility of the antibody to the epitope.

The PrP^C profiles of brain tissues result from overlapped PrP^C proteins but consist of heterogeneous subtypes because they can be differentiated into highly soluble and slightly soluble protein fractions. Differential solubility of PrP^C proteins derived from several species and various brain regions was shown recently using SDS as detergent [Kuczius et al., 2009]. In contrast, PrP^C of plt seem to consist of one or only few subtypes which are mainly present in the highly soluble fraction.

The glycoprotein banding patterns observed with plt and brain tissue result from glycosylated and non-glycosylated full-length PrP^C. However, PrP^C can be cleaved by endogenous proteolysis leaving a stable core protein. In humans, two amino-truncated isoforms were described which migrate at 18 and 21–22 kDa [Jiménez-Huete et al., 1998]. The cleavage sites are located at amino acids 110–112 and at residues 80–100 and the truncated isoforms are referred to as C1 and C2, respectively. The amino-truncated protein band C2, with a molecular mass of approximately 21–22 kDa, was identified using mab Pri308. The C1 fragment was determined with high-signal intensity and the C2 fragment with lower intensity using mab 12F10, which recognizes the core protein. These data indicate that plt PrP^C has restriction sites for endogenous proteases similar to PrP^C in brain. Blood and brain are very different materials which could easily contribute to divergent banding profiles and truncation products. Truncation occurs under natural conditions and may also arise by performance of protein preparation techniques so that the signals of the full-length and truncated isoforms are not quantified in this study.

Platelet PrP^C seem to be modified in a different way to that in brain. With regard to a PrP^{Sc} assembly in the cell, several aspects should be considered. These are the PrP^C expression levels, the primary PrP^C protein structure and the PrP^C glycosylation state. Infectivity of PrP^{Sc} in blood of scrapie-infected hamsters is associated with cells having no or only a very-low expression of PrP^C [Holada and Vostal, 2000]. The PrP^C patterns in brain tissues are highly heterogeneous as a result of full-length, fragmented and amino-terminal truncated proteins as demonstrated using 2D gel-electrophoresis [Pan et al., 2002]. Since the primary protein structure is the key parameter for the conversion [Taraboulos et al., 1990], many *N*-linked glycosylation profiles with a large diversity in the *N*-linked sugar chains interlinked to the PrP^C isoforms (Rudd et al., 1999) may also play an important role in the modulation of the conversion. This has been shown in vitro and in vivo experiments [Priola and Lawson 2001; Tuzi et al., 2008]. Biochemical conversion from PrP^C to PrP^{Sc} would then occur predominantly at locations where PrP^C is expressed in the appropriate form. This interpretation is supported by the finding that two or more PrP^{Sc} patterns coexist in humans and mice [Asante et al., 2002; Polymenidou et al., 2005; Yull et al., 2006]. The PrP^{Sc} types are present in low amounts which strengthen the hypothesis that only distinct PrP^C phenotypes can interact with high efficiency with distinct PrP^{Sc} subspecies.

According to this hypothesis, the determination of less diverse PrP^C types in plt may help to explain why only the transmission of the variant form of CJD (vCJD) and not the sporadic CJD (sCJD) has been observed. The PrP^C types may be more susceptible to the BSE and vCJD agents than to other isoforms. However, using the in vitro protein misfolding cyclic amplification technique for amplification and detection of traces of PrP^{Sc} proteins, PrP^C derived from human plt can be converted to PrP^{Sc} using sources of PrP^C from subjects with both subtypes of the disease, variant, and sporadic CJD [Jones et al., 2009]. Other parameters may also have an important influence on the infectivity of PrP^{Sc}. Among these are the different components and receptors present on blood cells such as heparan sulphate [Horonchik et al., 2005] and lipoproteins in plasma [Safar et al., 2006]. In addition, modified glycosylation patterns of PrP^C can induce a change in the PrP^{Sc} glycoprofile [Vorberg and Priola 2002].

In summary, our data demonstrate distinct glycoprotein profiles of PrP^C obtained from plt which clearly differ from PrP^C derived from brain tissues. Plt exhibit single or only a few types of PrP^C which is in contrast to PrP^C in brain which shows more diversity in the glycoprotein profiles. Furthermore, the glycosylation states and grades of plt PrP^C seem to be in a way different to that in brains. However, the physiological and functional significance of PrP^C in plt and for the transfer of infectivity of prion disease still remains to be determined.

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REFERENCES

Aguzzi A, Baumann F, Bremer J. 2008. The prion's elusive reason for being. Ann Rev Neurosci 31:439–477.

Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL, Welch J, Hill AF, Lloyd SE, Wadsworth JD, Collinge J. 2002. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. EMBO J 21:6358–6366.

Barclay GR, Houston EF, Halliday SI, Farquhar CF, Turner ML. 2002. Comparative analysis of normal prion protein expression on human, rodent, and ruminant blood cells by using a panel of prion antibodies. Transfusion 42:517–526.

Bendheim PE, Brown HR, Rudelli RD, Scala LJ, Goller NL, Wen GY, Kascsak RJ, Cashman NR, Bolton DC. 1992. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. Neurology 42:149–156.

Beringue V, Mallinson G, Kaisar M, Tayebi M, Sattar Z, Jackson G, Anstee D, Collinge J, Hawke S. 2003. Regional heterogeneity of cellular prion protein isoforms in the mouse brain. Brain 126:2065–2073.

Brown P, Cervenakova L, McShane LM, Barber P, Rubenstein R, Drohan WN. 1999. Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt–Jakob disease in humans. Transfusion 39:1169–1178.

Cashman NR, Loertscher R, Nalbantoglu J, Shaw I, Kascsak RJ, Bolton DC, Bendheim PE. 1990. Cellular isoform of the scrapie agent protein participates in lymphocyte activation. Cell 61:185–192.

Cervenakova L, Yakovleva O, McKenzie C, Kolchinsky S, McShane L, Drohan WN, Brown P. 2003. Similar levels of infectivity in the blood of mice infected with human-derived vCJD and GSS strains of transmissible spongiform encephalopathy. Transfusion 43:1687–1694.

Choi EM, Geschwind MD, Deering C, Pomeroy K, Kuo A, Miller BL, Safar JG, Prusiner SB. 2009. Prion proteins in subpopulations of white blood cells from patients with sporadic Creutzfeldt–Jakob disease. Lab Invest 89:624–635.

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. 1996. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 383:685–690.

Demart S, Fournier JG, Creminon C, Frobert Y, Lamoury F, Marce D, Lasmezas C, Dormont D, Grassi J, Deslys JP. 1999. New insight into abnormal prion protein using monoclonal antibodies. Biochem Biophys Res Comm 265:652–657.

Dodelet VC, Cashman NR. 1998. Prion protein expression in human leukocyte differentiation. Blood 91:1556–1561.

Durig J, Giese A, Schulz-Schaeffer W, Rosenthal C, Schmucker U, Bieschke J, Duhrsen U, Kretzschmar HA. 2000. Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes. Br J Haematol 108:488–495.

Fagge T, Barclay GR, MacGregor I, Head M, Ironside J, Turner M. 2005. Variation in concentration of prion protein in the peripheral blood of patients with variant and sporadic Creutzfeldt–Jakob disease detected by dissociation enhanced lanthanide fluoroimmunoassay and flow cytometry. Transfusion 45:504–513.

Holada K, Vostal JG. 2000. Different levels of prion protein (PrP^C) expression on hamster, mouse and human blood cells. Br J Haematol 110:472–480.

Holada K, Vostal JG, Theisen PW, MacAuley C, Gregori L, Rohwer RG. 2002. Scrapie infectivity in hamster blood is not associated with platelets. J Virol 76:4649–4650.

Holada K, Glierova H, Simak J, Vostal JG. 2006. Expression of cellular prion protein on platelets from patients with gray platelet or Hermansky–Pudlak syndrome and the protein's association with alpha-granules. Haematologica 91:1126–1129.

Holada K, Simak J, Brown P, Vostal JG. 2007. Divergent expression of cellular prion protein on blood cells of human and nonhuman primates. Transfusion 47:2223–2232.

Horonchik L, Tzaban S, Ben-Zaken O, Yedidia Y, Rouvinski A, Papy-Garcia D, Barritault D, Vlodavsky I, Taraboulos A. 2005. Heparan sulfate is a cellular receptor for purified infectious prions. J Biol Chem 280:17062–17067.

Houston F, Foster JD, Chong A, Hunter N, Bostock CJ. 2000. Transmission of BSE by blood transfusion in sheep. Lancet 356:999–1000.

Houston F, McCutcheon S, Goldmann W, Chong A, Foster J, Sisó S, González L, Jeffrey M, Hunter N. 2008. Prion diseases are efficiently transmitted by blood transfusion in sheep. Blood 112:4739–4745.

Jiménez-Huete A, Lievens PM, Vidal R, Piccardo P, Ghetti B, Tagliavini F, Frangione B, Prelli F. 1998. Endogenous proteolytic cleavage of normal and disease-associated isoforms of the human prion protein in neural and non-neural tissues. Am J Pathol 153:1561–1572.

Jones M, Head MW, Connolly JG, Farquhar CF, Hornsey VS, Pepper DS, MacGregor IR. 2005. Purification of normal cellular prion protein from human platelets and the formation of a high molecular weight prion protein complex following platelet activation. Biochem Biophys Res Comm 335:48–56.

Jones M, Peden AH, Yull H, Wight D, Bishop MT, Prowse CV, Turner ML, Ironside JW, MacGregor IR, Head MW. 2009. Human platelets as a substrate source for the in vitro amplification of the abnormal prion protein (PrP) associated with variant Creutzfeldt–Jakob disease. Transfusion 49:376–384.

Jouvin-Marche E, Attuil-Audenis V, Aude-Garcia C, Rachidi W, Zabel M, Podevin-Dimster V, Siret C, Huber C, Martinic M, Riondel J, Villiers CL, Favier A, Naquet P, Cesbron JY, Marche PN. 2006. Overexpression of cellular prion protein induces an antioxidant environment altering T cell development in the thymus. J Immunol 176:3490–3497.

Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, Wisniewski HM, Diringer H. 1987. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J Virol 61:3688–3693.

Krasemann S, Groschup M, Hunsmann G, Bodemer W. 1996. Induction of antibodies against human prion proteins (PrP) by DNA-mediated immunization of PrP0/0 mice. J Immunol Methods 199:109–118.

Kretzschmar HA, Prusiner SB, Stowring LE, DeArmond SJ. 1986. Scrapie prion proteins are synthesized in neurons. Am J Pathol 122:1–5.

Kuczius T, Grassi J, Karch H, Groschup MH. 2007a. Binding of N- and C-terminal anti-prion protein antibodies generates distinct phenotypes of cellular prion proteins (PrPC) obtained from human, sheep, cattle and mouse. FEBS J 274:1492–1502.

Kuczius T, Koch R, Keyvani K, Karch H, Grassi J, Groschup MH. 2007b. Regional and phenotype heterogeneity of cellular prion proteins in the human brain. Eur J Neurosci 25:2649–2655.

Kuczius T, Karch H, Groschup MH. 2009. Differential solubility of cellular prions is associated in manifold phenotypes. Mol Cell Neurosci 42:226–233.

Lasmézas CI. 2003. Putative functions of PrP(C). Br Med Bull 66:61–70.

Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, Will RG. 2004. Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion. Lancet 363:417–421.

MacGregor I, Hope J, Barnard G, Kirby L, Drummond O, Pepper D, Hornsey V, Barclay R, Bessos H, Turner M, Prowse C. 1999. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang. 77:88–96.

Morel N, Simon S, Frobert Y, Volland H, Mourton-Gilles C, Negro A, Sorgato MC, Créminon C, Grassi J. 2004. Selective and efficient immunoprecipitation of the disease-associated form of the prion protein can be mediated by

nonspecific interactions between monoclonal antibodies and scrapie-associated fibrils. J Biol Chem 279:30143–30149.

Pan T, Li R, Wong BS, Liu T, Gambetti P, Sy MS. 2002. Heterogeneity of normal prion protein in two-dimensional immunoblot: Presence of various glycosylated and truncated forms. J Neurochem 81:1092–1101.

Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. 2004. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet 364:527–529.

Polymenidou M, Stoeck K, Glatzel M, Vey M, Bellon A, Aguzzi A. 2005. Coexistence of multiple PrPSc types in individuals with Creutzfeldt–Jakob disease. Lancet Neurol 4:805–814.

Priola SA, Lawson VA. 2001. Glycosylation influences cross-species formation of protease-resistant prion protein. EMBO J 20:6692–6699.

Prusiner SB. 1998. Prions. Proc Natl Acad Sci USA 95:13363–13383.

Robertson C, Booth SA, Beniac DR, Coulthart MB, Booth TF, McNicol A. 2006. Cellular prion protein is released on exosomes from activated platelets. Blood 107:3907–3911.

Rudd PM, Endo T, Colominas C, Groth D, Wheeler SF, Harvey DJ, Wormald MR, Serban H, Prusiner SB, Kobata A, Dwek RA. 1999. Glycosylation differences between the normal and pathogenic prion protein isoforms. Proc Natl Acad Sci USA 96:13044–13049.

Safar JG, Wille H, Geschwind MD, Deering C, Latawiec D, Serban A, King DJ, Legname G, Weisgraber KH, Mahley RW, Miller BL, DeArmond SJ, Prusiner SB. 2006. Human prions and plasma lipoproteins. Proc Natl Acad Sci USA 103:11312–11317.

Sales N, Rodolfo K, Hassig R, Faucheux B, Di Giamberardino L, Moya KL. 1998. Cellular prion protein localization in rodent and primate brain. Eur J Neurosci 10:2464–2471.

Taraboulos A, Rogers M, Borchelt DR, McKinley MP, Scott M, Serban D, Prusiner SB. 1990. Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. Proc Natl Acad Sci USA 87:8262–8266.

Taylor DM, Fernie K, Steele PJ. 1999. Infectivity of scrapie prions. Mol Med 5:701.

Tuzi NL, Cancellotti E, Baybutt H, Blackford L, Bradford B, Plinston C, Coghill A, Hart P, Piccardo P, Barron RM, Manson JC. 2008. Host PrP glycosylation: A major factor determining the outcome of prion infection. PLoS Biol 6(4): e100.

Vorberg I, Priola SA. 2002. Molecular basis of scrapie strain glycoform variation. J Biol Chem 277:6775–36781.

Yull HM, Ritchie DL, Langeveld JP, van Zijderveld FG, Bruce ME, Ironside JW, Head MW. 2006. Detection of type 1 prion protein in variant Creutzfeldt–Jakob disease. Am J Pathol 168:151–157.