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Intra- and Interspecies Interactions between Prion Proteins and Effects of Mutations and Polymorphisms

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Recently, crystallization of the prion protein in a dimeric form was reported. Here we show that native soluble homogenous FLAG-tagged prion proteins from hamster, man and cattle expressed in the baculovirus system are predominantly dimeric. The PrP/PrP interaction was confirmed in Semliki Forest virus-RNA transfected BHK cells co-expressing FLAG- and oligohistidine-tagged human PrP. The yeast two-hybrid system identified the octarepeat region and the C-terminal structured domain (aa90-aa230) of PrP as PrP/PrP interaction domains. Additional octarepeats identified in patients suffering from fCJD reduced (wtPrP *versus* PrP+9OR) and completely abolished (PrP+9OR *versus* PrP+9OR) the PrP/PrP interaction in the yeast two-hybrid system. In contrast, the Met/Val polymorphism (aa129), the GSS mutation Pro102Leu and the FFI mutation Asp178Asn did not affect PrP/PrP interactions. Proof of interactions between human or sheep and bovine PrP, and sheep and human PrP, as well as lack of interactions between human or bovine PrP and hamster PrP suggest that interspecies PrP interaction studies in the yeast two-hybrid system may serve as a rapid pre-assay to investigate species barriers in prion diseases.

Keywords: Dimeric prion protein / Interspecies interaction / Mutations / Polymorphism / PrP-PrP interaction domains / Semliki Forest virus / Yeast two-hybrid system.

Introduction

Prions are thought to be the infectious agents of transmissible spongiform encephalopathies (TSEs) (for review, see Weissmann and Aguzzi, 1997; Prusiner *et al.*, 1998; Lasmézas and Weiss, 2000). Several binding partners for the cellular form of the prion protein have been identified (for review Gauczynski *et al.*, 2001a), among them are molecular chaperones such as Hsp60 (Edenhofer *et al.*, 1996), protein X (Kaneko *et al.*, 1997) and the 37 kDa/67 kDa laminin receptor (Rieger *et al.*, 1997), which has been identified as the receptor for the cellular prion protein (Gauczynski *et al.*, 2001b) and demonstrated to be required for PrP^{Sc} propagation in neuronal cells (Leucht *et al.*, 2003; for a review see Leucht and Weiss, 2002).

One of the proposed models explaining the replication of prions is the protein-only hypothesis (Alper *et al.*, 1967; Griffith, 1967; Prusiner, 1982), which states that a PrP^{Sc} monomer interacts with a PrP^{c} monomer to form a PrP^{Sc}/PrP^{c} heterodimer. PrP^{Sc} then converts PrP^{c} to a PrP^{Sc} homodimer by changing its secondary/tertiary structure. Another model states that a nucleus or seed consisting of PrP^{Sc} molecules incorporates PrP^{c} monomers starting a nucleation-dependent polymerization or crystal seed reaction in which the PrP^{c} monomers become converted to PrP^{Sc} molecules (Lansbury and Caughey, 1995). Both models result in an oligo-/multimerization process finally leading to PrP^{Sc} aggregation.

PrP dimers (for review see Gauczynski *et al.*, 2001a) could play an essential role in this conversion process and have been characterized as an intermediate state during PrP-multimerization as analyzed by fluorescence correlation spectroscopy (FCS) (Post *et al.*, 1998; Jansen *et al.*, 2001). PrP dimers have been observed in N_2a cells and in scrapie-infected hamster brains (Priola *et al.*, 1995). Molecular modeling suggested the existence of PrP/PrP dimers (Warwicker and Gane, 1996), which might be involved in PrP interspecies transmission (Warwicker, 1997). The existence of a monomer-dimer equilibrium of partially purified PrP^{c} from cattle has been shown (Meyer *et al.*, 2000). Recently, crystallization of dimeric PrP has been reported involving domain swapping of α-helical structures (Knaus *et al.*, 2001). Very recently, a soluble and dimeric PrP fused to immunoglobulin Fcγ (PrP-Fc_2) was shown to delay PrP^{Sc} accumulation in mice (Meier *et al.*, 2003). Here we show by size exclusion chromatography that recombinant FLAG-tagged PrP from hamster, human and cattle, purified to homogeneity from the baculovirus system, elute predominantly as dimers under native conditions. In the presence of DTT, the monomeric PrP form was marginally increased suggesting that disulfide bonds do not contribute to dimer formation. We confirmed the PrP/PrP interaction in BHK cells co-expressing oligohistidine and FLAG-tagged prion proteins using recombinant Semliki Forest Virus RNAs. Employing the yeast two-hybrid system, in which the PrP/PrP interaction was further confirmed, we identified both the octarepeat region and the carboxy terminus of the prion protein (PrP90-230) as PrP/PrP interaction domains.

Defined mutations within the *Prn*-p gene lead to famil-

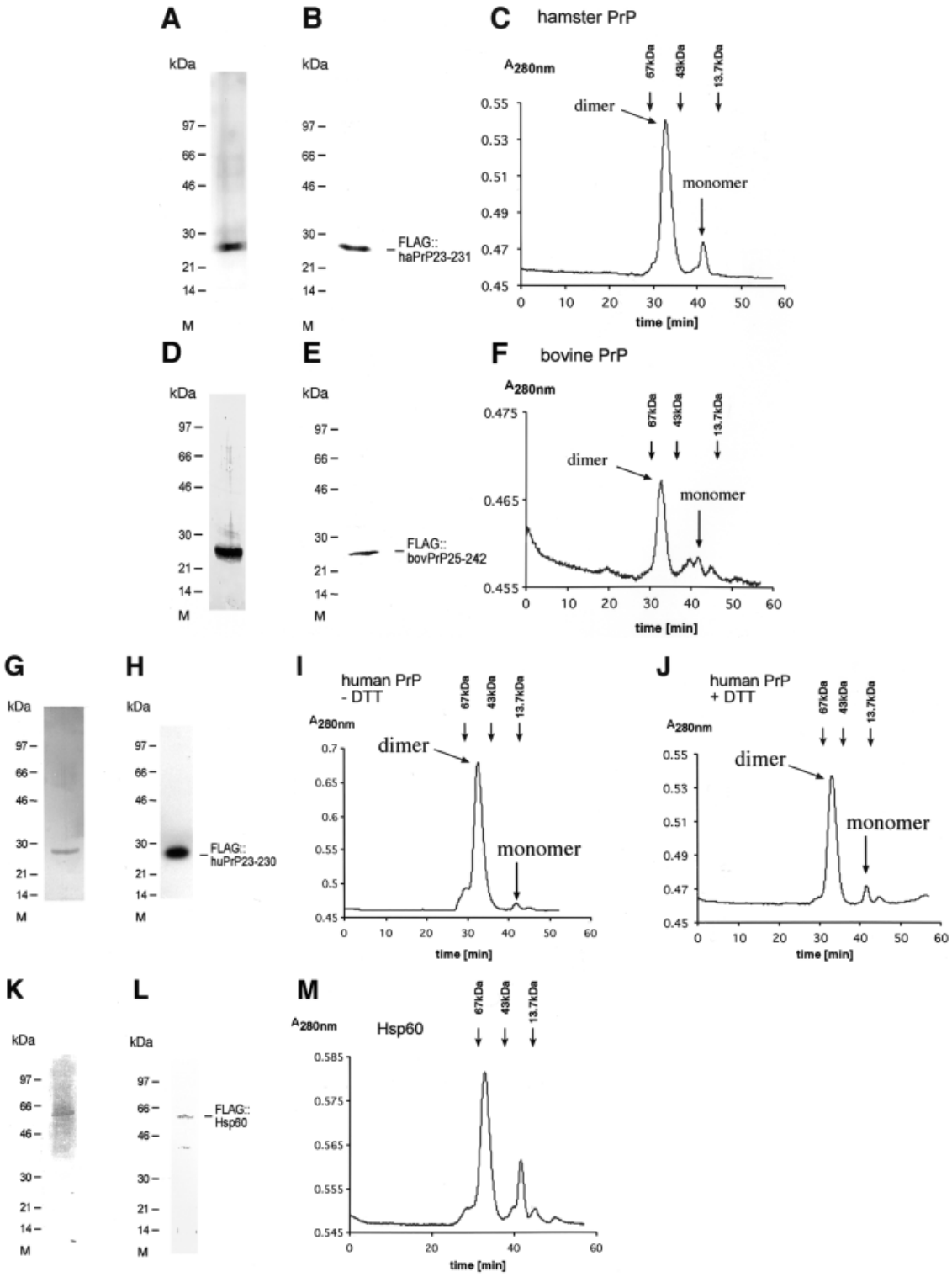

Fig. 1 Analysis of Recombinant FLAG::PrP from Hamster, Cattle and Man and FLAG::Hsp60 under Native and Denaturing Conditions. Four hundred ng of purified FLAG::haPrP23-231 were analyzed by SDS-PAGE (12.5% PA) followed by silver staining of the gel (A) and Western blotting (B) employing the monoclonal 3B5 antibody. (C) Six µg of non-denatured FLAG::haPrP23-231 were analyzed by size exclusion chromatography (SEC) on a Superose 12 PC 3.2/30 column (Amersham Pharmacia). Marker proteins (LMW calibration kit) are indicated. Six hundred ng of purified FLAG::bovPrP25-242 were analyzed by SDS-PAGE (12.5% PA) followed by silver staining of the gel (D) and Western blotting (E) employing the 3 B5 antibody. (F) Four µg of non-denatured FLAG::bovPrP25-242 were analyzed by SEC as described above. Four hundred ng of FLAG::huPrP23-230 were analyzed by SDS-PAGE (12.5% PA) followed by silver staining of the gel (G) and Western blotting employing the JB007 antibody (H). (I) Eight µg of non-denatured FLAG::huPrP23-230 were analyzed by SEC in the absence of DTT as described above. (J) Six µg of FLAG::huPrP23-230 were analyzed by SEC after denaturing with DTT. Three hundred ng of purified FLAG::HSP60 were analyzed by SDS-PAGE (12.5% PA) followed by silver staining of the gel (K) and Western blotting (L) employing an Hsp60-specific antibody. (M) Four µg of non-denatured FLAG::Hsp60 were analyzed by SEC as described above.

ial Creutzfeldt-Jakob Disease (fCJD), Gerstmann-Sträussler-Scheinker (GSS) Syndrome and fatal-familial insomnia (FFI) (for review see Lasmézas and Weiss, 2000). A series of mutations affect the octarepeat region of the prion protein. fCJD patients encompassing two (Goldfarb *et al.*, 1993), four (Campbell *et al.*, 1996), five (Goldfarb *et al.*, 1991), six (Owen *et al.*, 1990), seven (Goldfarb *et al.*, 1991), eight (Goldfarb *et al.*, 1991) and nine additional octarepeats (Owen *et al.*, 1992) have been described. All these patients are heterozygous regarding these mutations (Majtenyi *et al.*, 2000). The mutation proline 102 to leucine leads to GSS (Goldgaber *et al.*, 1989). The polymorphism at position 129 of the human prion protein determines whether an organism suffers from FFI (position 178 Asp to Asn, together with methionine at position 129), fCJD (position 178 Asp to Asn, together with valine at position 129) or remains healthy (178 Asp and 129 Met or 129 Val) (Tateishi *et al.*, 1995, and references therein). Each patient suffering from nvCJD investigated so far was homozygous for methionine at position 129 (Zeidler *et al.*, 1997; Hill *et al.*, 1999). We investigated whether relevant mutations and polymorphisms within the *Prn*-p gene affect the PrP/PrP interaction behavior. Mutated PrP proteins with alterations in the octarepeat region, the first PrP/PrP binding domain, encompassing two, five and nine additional octarepeats reduced the PrP/PrP interaction when tested against wild-type PrP, and completely abolished the PrP/PrP interaction when PrP+5OR or PrP+9OR were tested against each other. Point mutations located in the second PrP/PrP interaction domain (90-230), however, leading to amino acid substitutions at positions 102 (proline to leucine), 129 (methionine to valine) and 178 (aspartate to asparagine), respectively, did not influence the PrP/PrP interaction behavior as assayed in the yeast two-hybrid system, suggesting a pathogenic mechanism different from that induced by the additional octarepeats. Finally, we investigated interspecies interactions of prion proteins of different species including man, cattle, sheep and hamsters in the yeast two-hybrid system, and suggest that this system might be a useful and rapid pre-assay to investigate species barriers in prion diseases.

Results

Recombinant Human, Bovine and Hamster PrP Are Dimeric under Native Conditions

FLAG-tagged human, bovine and hamster PrP were synthesized in Sf9 cells infected with recombinant baculoviruses and purified to homogeneity by anti-FLAG antibody chromatography. The homogeneous PrP from hamster, cattle and man revealed a molecular mass of approx. 27 kDa under denaturing and reducing conditions on an SDS-polyacrylamide gel (Figure 1A, D and G) and was recognized by PrP-specific antibodies (Figure 1B, E and H). Under native conditions, however, FLAG-tagged hamster, bovine and human PrP revealed molecular masses of 53, 54 and 53 kDa, respectively, and to a minor extent molecular masses of 24, 25 and 24 kDa, respectively, as determined by size exclusion chromatography (Figure 1C, F and I), demonstrating that PrP from these three species are predominantly dimeric under native conditions. The measured values are close to the values derived from the individual amino acid sequences, which are 23.9, 24.5 and 23.7 kDa, respectively, for hamster, bovine and human PrP. In the presence of DTT, the proportion of the monomeric form of human PrP was increased from 1.9 to 6.6%, whereas the proportion of the dimeric form decreased from 98.1 to 93.4% (Figure 1J), suggesting that intermolecular disulfide bonds do not, or only marginally, contribute to PrP dimer formation. Analysis of the FLAG-tagged prion proteins of all three species on native PA gels in the absence and presence of β-mercaptoethanol resulted in no difference in the migration pattern (data not shown), confirming the result from the size exclusion chromatography that intermolecular disulfide bonds do not, or only marginally, contribute to PrP dimer formation. The FLAG-tagged heat shock protein Hsp60 synthesized in the baculovirus system (Figure 1K, L) was monomeric under native conditions as measured by size exclusion chromatography (Figure 1M), demonstrating that the FLAG tag is not responsible for the dimerization behavior of FLAG- tagged PrP. The strong peak representing a molecule of 40 kDa is a breakdown product of the FLAG- tagged Hsp60, which is also visible in the Western blot (Figure 1L), whereas the small peak at a molecular mass of 13.7 kDa reveals a breakdown product that is too small in amount to be visible by Western blotting (Figure 1L).

The FLAG-tagged dimeric prion proteins from hamster, man and cattle revealed proteinase K (pK) sensitivity at 4 µg/ml pK incubated for 60 min at 37 °C (data not shown) suggesting that all three recombinant proteins are non-infectious. Recombinant monomeric prion proteins from hamster, man and cattle (purchased from Prionics) also showed total pK sensitivity under the same experimental conditions (data not shown), demonstrating that monomeric and dimeric prion proteins do not differ with respect to proteinase K sensitivity.

PrP/PrP Interaction in Recombinant Semliki Forest Virus (SFV) RNA Transfected BHK Cells Co-Expressing Oligohistidine- and FLAG-Tagged PrP

In order to confirm the PrP/PrP interaction in a eukaryotic system, we chose BHK cells co-expressing highly glycosylated oligohistidine- and FLAG-tagged human prion proteins after transfection with recombinant Semliki Forest Virus RNAs. The protein/protein interaction was investigated by pull-down assays with immobilized oligohistidine-tagged PrP on nickel columns followed by the detection of the interacting FLAG-tagged protein with an anti-FLAG antibody. After co-expression of PrP227-oligohistidine-228–253 and PrP227-FLAG-228–253,

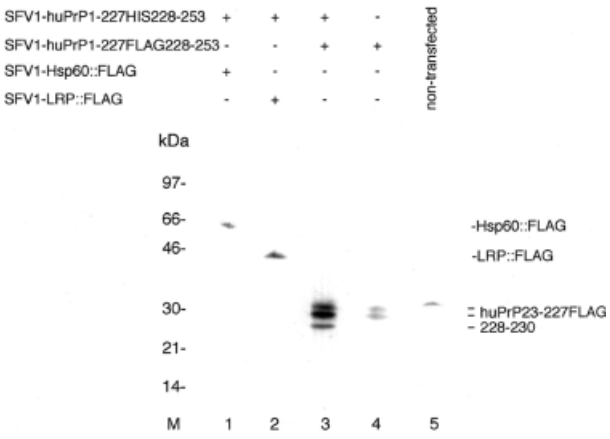

Fig. 2 Analysis of PrP/PrP Interactions in BHK Cells Co-Transfected with Recombinant Semliki Forest Virus RNAs.
Extracts of total protein from BHK cells either non-transfected (lane 5), transfected with SVF1-huPrP1-227FLAG228-253 (lane 4) or co-transfected with SVF1-huPrP1-227FLAG228-253 and SVF1-huPrP1-227HIS228-253 (lane 3), SVF1-huPrP1-227HIS228-253 and SFV1-LRP::FLAG (lane 2) or SVF1-huPrP1-227HIS228-253 and SFV1-Hsp60::FLAG (lane 1) were harvested 48 h post-transfection, purified by IMAC, analyzed on a 12% PAA-SDS gel, blotted and developed with the monoclonal anti-FLAG antibody (M2).

the non-, mono-, and diglycosylated forms of FLAG-tagged PrP were detectable (Figure 2, lane 3), demonstrating the interaction of both prion proteins in BHK cells. Expression of FLAG-tagged PrP alone resulted in a weak background binding of the mono- and diglycosylated forms of the prion protein to the nickel column due to the histidine-rich octarepeat region of PrP (lane 4). Here the sugars seem to have an effect on the residual nickel binding since the non-glycosylated band does not interact with the nickel column. As positive controls the PrP interacting proteins 37 kDa laminin receptor precursor (LRP; Rieger *et al.*, 1997; Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001; Leucht *et al.*, 2003; Simoneau *et al.*, 2003) and Hsp60 (Edenhofer *et al.*, 1996) (lane 1), both tagged with FLAG, were used. Both proteins bound to oligohistidine-tagged PrP (lane 1 and 2, respectively). As a negative control PrP did not interact with LRPdelBD::FLAG lacking the direct PrP binding domain expressed in CHO-S-745 cells (Hundt *et al.*, 2001; Figure 4, panel K).

PrP/PrP Interaction and Identification of PrP/PrP Interaction Domains by Yeast Two-Hybrid Analyses

Co-expression of human PrP tagged to the highly soluble GST in bait and prey positions of the yeast two-hybrid system (Gyuris *et al.*, 1993) resulted in a strong interaction of both proteins (Figure 3, row 3), confirming the PrP/PrP interaction observed in recombinant SFV-RNA transfected BHK cells. GST did not interact with itself or with GST::PrP^{c} (Figure 3, rows 1 and 2, respectively). Next, we investigated which regions of the prion protein are involved in the PrP/PrP interaction process. The highly flexible unstructured octarepeat region of PrP, also known as the proline/glycine-rich region (Donne *et al.*, 1997; Riek *et al.*, 1997), which has been shown to bind copper *in vivo* (Brown *et al.*, 1997), consists of five (six in cattle) repeats of a stretch of eight amino acids (PHGGG-WGQ). Co-expression of the human PrP octarepeat domain in both positions of the yeast two-hybrid system resulted in a strong interaction between the two truncated PrP proteins (Figure 3, row 5). The ultimate amino-terminus of PrP (aa23-50), however, failed to interact with itself (Figure 3, row 6), demonstrating that this region of PrP is not involved in the PrP/PrP interaction. Deletion of the octarepeat domain resulted in a weaker interaction signal (Figure 3, row 4), suggesting the existence of a second PrP/PrP interaction domain. Co-expression of PrP90-230 in both bait and prey positions of the yeast two-hybrid system indeed resulted in a strong interaction signal (Figure 3, row 7). In summary, these data demonstrate that the octarepeat region and PrP90-230 contribute to PrP/PrP interactions.

Additional Octarepeats Located in the First PrP/PrP Interaction Domain Impede PrP/PrP Interaction

Since the octarepeat region represents a PrP/PrP interaction domain, we investigated whether additional octarepeats identified in familial CJD patients might influence the PrP interaction behavior. When human PrP encompassing two (Figure 4, row 7), five (row 8) and nine (row 9) additional octarepeats was expressed in bait *versus* wild-type PrP in the prey position of the yeast two-hybrid system, the PrP/PrP interaction was only slightly diminished. This situation mimics the heterozygous state in all patients investigated so far expressing the mutated PrP on one allele and the wild-type PrP from the other allele. However, when mutated human prion proteins encompassing two (Figure 4, row 4), five (row 5) and nine (row 6) additional octarepeats were co-expressed in both positions of the yeast two-hybrid system, reflecting a thus far hypothetical case of a patient homozygous for this *Prn*-p mutation, the PrP/PrP interaction was strongly reduced with two additional octarepeats (row 4) and completely abolished with five (row 5) and nine (row 6) additional octarepeats.

The Mutations Pro102Leu (GSS), Asp178Asn (FFI) and the Polymorphism Met129Val Do Not Influence the PrP/PrP Interaction

We investigated the polymorphism Met-Val at position 129, the mutation Pro-Leu at position 102 and the mutation Asp-Asn at position 178 (polymorphism aa129 methionine) of the human prion protein with respect to their influence on the PrP/PrP interaction behavior. Neither this polymorphism nor the mutation at position 102 expressed as bait *versus* wild-type PrP as prey (Figure 5, lanes 6 and 4), or both expressed in both bait and prey positions (Figure 5, lanes 7 and 5), reflecting the heterozygous and homozygous states, respectively, affected the PrP/PrP interaction behavior. In addition, the FFI-

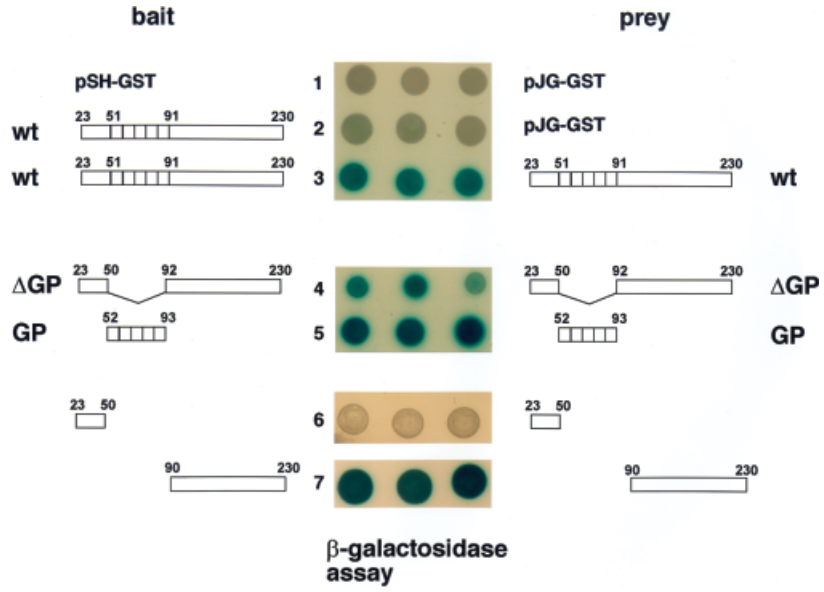

Fig. 3 Identification of PrP/PrP Interaction Domains in the Yeast Two-Hybrid System (*S. cerevisiae*).
Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (row 3), pJG-GST::PrPΔGP (row 4), pJG-GST::GP52-93 (row 5), pJG-GST::PrP23-50 (row 6) and pJG-GST::PrP90-230 (row 7) as well as the bait plasmids pSH-GST (row 1), pSH-GST::huPrP23-230 (rows 2 and 3), pSH-GST::PrPΔGP (row 4), pSH-GST::PrP52-93 (row 5), pSH-GST::PrP23-50 (row 6) and pSH-GST::PrP90-230 (row 7). Each of three transformants were resuspended in TE, dotted on X-gal-supplemented plates and incubated at 30 °C for 3 days (β-galactosidase assay).

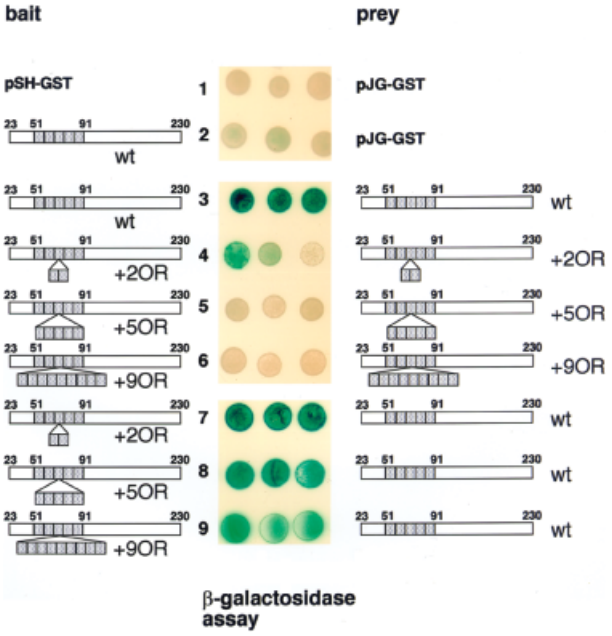

Fig. 4 Influence of Additional Octarepeats on the PrP/PrP Interaction Behavior.
Yeast cells encompassing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (rows 3, 7, 8 and 9), pJG-GST::PrP+2OR (row 4), pJG-GST::PrP+5OR (row 5), pJG-GST::PrP+9OR (row 6) as well as the bait plasmids pSH-GST (row 1), pSH-GST::PrP23-230 (rows 2 and 3), pSH-GST::PrP+2OR (rows 4 and 7), pSH-GST::PrP+5OR (rows 5 and 8), pSH-GST::PrP+9OR (rows 6 and 9) (β-galactosidase assay).

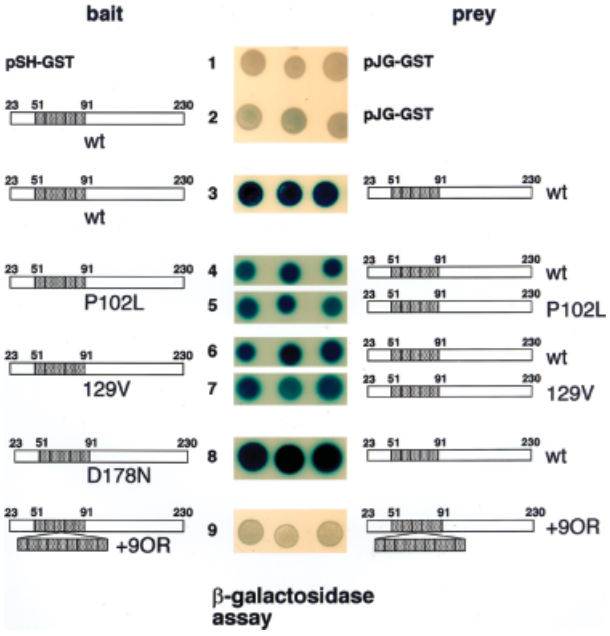

Fig. 5 Influence of the GSS Mutation P102L, the Polymorphism M129V and the Mutation D178N on the PrP/PrP Interaction Behavior Analyzed in the Yeast Two-Hybrid System.
Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (rows 3, 4, 6 and 8), pJG-GST::PrP-P102L (row 5), pJG-GST::PrP-M129V (row 7), and pJG-GST::PrP+9OR (row 9) as well as the bait plasmids pSH-GST (row 1), pSH-GST::PrP23-230 (rows 2 and 3), pSH-GST::PrP-P102L (rows 4 and 5), pSH-GST::PrP-M129V (rows 6 and 7), pSH-GST::PrP-D178N (row 8) and pSH-GST::PrP+9OR (row 9) (β-galactosidase assay).

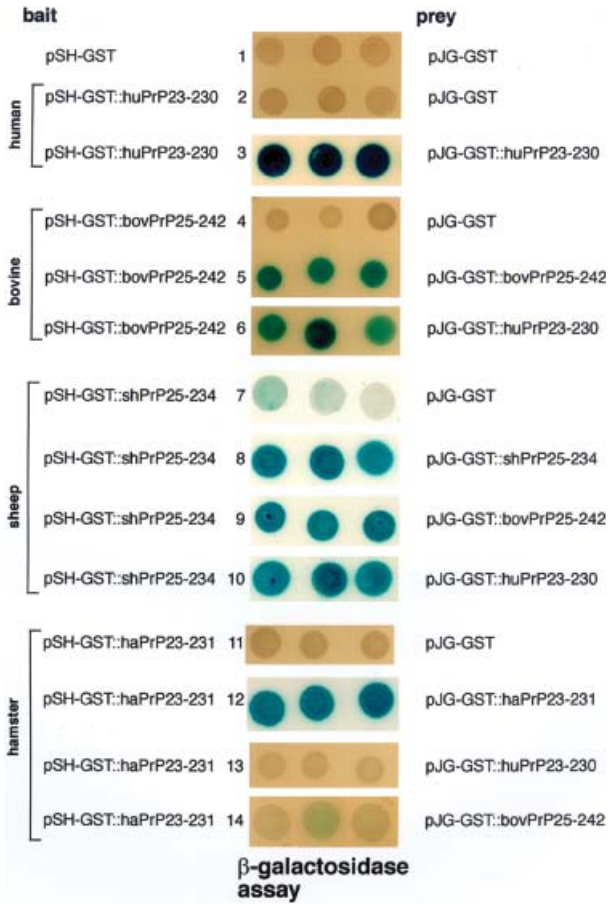

Fig. 6 Interaction Analysis of PrP of Different Species in the Yeast Two-Hybrid System.
The bait plasmids pSH-GST (row 1), pSH-GST::huPrP23-230 (rows 2 and 3), pSH-GST::bovPrP25-242 (rows 4–6), pSH-GST::shPrP25-234 (AQ) (rows 7–10) and pSH-GST::haPrP (rows 11–14) were co-transformed with the reporter plasmid pSH18-34 and the prey plasmids pJG-GST (rows 1, 2, 4, 7 and 11), pJG-GST::huPrP23-230 (rows 3, 6, 10 and 13), pJG-GST::bovPrP25-242 (rows 5, 9 and 14) and pJG-GST::shPrP25-234 (AQ) (row 8) (β-galactosidase assay).

related mutation 178 Asp to Asn (aa129 methionine) did not influence the PrP/PrP interaction behavior when expressed in bait *versus* wild-type PrP in the prey position (heterozygous case; Figure 5, lane 8). We conclude that both mutations together with the polymorphism, which all reside within PrP90-230, have no influence on the PrP/PrP interaction behavior. Thus PrP dimers can be formed in all familial CJD (including cases due to additional octarepeats since they are exclusively heterozygous), GSS and FFI cases investigated.

Interaction of Prion Proteins of Different Species in the Yeast Two-Hybrid System

In order to investigate whether prion proteins of different species interact with each other, we verified the interaction between prion proteins of different species in the yeast two-hybrid system. As already observed with human PrP (Figure 3), bovine PrP (Figure 6, row 5), ovine PrP (AQ) (Figure 6, row 8) and hamster PrP (Figure 6, row12) also interact with each other. Regarding interspecies interactions, human PrP interacts with bovine PrP (Figure 6, row 6) and ovine PrP (AQ) (Figure 6, row 10), but not with hamster PrP (Figure 6, row 13). Bovine PrP shows an interaction with ovine PrP (Figure 6, row 9) but no interaction with hamster PrP (Figure 6, row 14). For specificity controls we verified that no PrP species binds to GST (Figure 6, rows 2, 4, 7 and 11).

Discussion

According to the protein-only hypothesis (Prusiner, 1982) and the nucleation-dependent polymerization model (Lansbury and Caughey, 1995), PrP^{c} converts into PrP^{Sc} by either a PrP^{Sc} monomer (Prusiner, 1982) or a PrP^{Sc} seed (Lansbury and Caughey, 1995). Recently, PrP dimers have been characterized as an intermediate state during the PrP-oligo- and multimerization processes analyzed by fluorescence correlation spectroscopy (FCS) (Post *et al.*, 1998). PrP dimers consisting of α-helical PrP monomers were only stable for less than a minute but PrP dimers consisting of mainly β-sheeted monomers have been found to be stable for about 10 minutes (Post *et al.*, 1998). Recently, a soluble and stable α-helical intermediate of recombinant hamster PrP (90-231) was identified by size exclusion chromatography and chemical cross-linking (Jansen *et al.*, 2001). PrP dimers have also been described in uninfected mouse neuroblastoma cells (Priola *et al.*, 1995) with an approximate molecular mass of 60 kDa. Similar 60 kDa PrP molecules were identified in scrapie-infected hamster brains but not in uninfected brains (Priola *et al.*, 1995). These authors suggested that the 60 kDa dimeric PrP might contribute to the conversion of protease-sensitive to protease K-resistant PrP. Very recently, we generated a covalently-linked dimer of the prion protein (Riley *et al.*, 2002). This highly glycosylated dimer is transported to the plasma membrane and reveals proteinase K sensitivity, suggesting that the dimer lacks endogenous infectivity.

Dimerization of Recombinant Prion Proteins from Human, Cattle and Hamster under Native Conditions

A monomer/dimer equilibrium of partially-purified PrP^{c} from cattle has been described previously (Meyer *et al.*, 2000). More recently, crystallization of the dimeric recombinant PrP has been reported, involving domain swapping of the C-terminal helix 3 and rearrangement of the disulfide bond (Knaus *et al.*, 2001). We have shown here that recombinant full-length FLAG-tagged PrP from human, cattle or hamster purified from insect cells infected with recombinant baculoviruses exist predominantly in dimeric form under native conditions. Our recombinant PrP was purified under native conditions from the medium of insect cells infected with recombinant baculovirus-

es. Addition of DTT increases the monomeric form of PrP slightly, suggesting that disulfide bridges do not, or only marginally, participate in PrP dimer formation. We can exclude that the FLAG tag used for purification induces dimerization of our recombinant protein, since FLAG-tagged Hsp60 appeared to be solely monomeric.

All recombinant dimeric prion proteins of the three different species revealed proteinase K sensitivity, suggesting that these proteins lack infectivity. Since monomeric prion proteins from the same species (purchased from Prionics) showed the same proteinase K sensitivity, we conclude that monomeric and dimeric prion proteins do not differ with respect to proteinase K sensitivity and infectivity.

PrP/PrP Interactions in BHK Cells Transfected with Recombinant SFV-RNAs

In order to prove the PrP/PrP interaction in highly developed eukaryotic cells, we transiently co-expressed FLAG-tagged and oligohistidine-tagged PrP in BHK cells using the Semliki Forest Virus system. Employing pull down assays, we demonstrated the interaction of these highly glycosylated prion proteins in an evolutionarily highly developed cell system.

PrP/PrP Interactions and Identification of PrP/PrP Interaction Domains

Direct PrP/PrP interactions have not been reported so far. We employed the yeast two-hybrid system as a powerful tool for the detection of protein/protein interactions (Gyuris *et al.*, 1993). The yeast two-hybrid system is also useful for the identification of interaction domains of cytosolic (Lopez *et al.*, 2001) and membrane-associated proteins (Bowman *et al.*, 2000). Expression of PrP in bait and prey positions of the yeast two-hybrid system resulted in a direct interaction of both proteins. Expression of PrP truncations and deletion mutants in the same system identified the octarepeat region as one PrP/PrP interaction domain with a copper binding capacity *in vivo* (Brown *et al.*, 1997) and an intrinsic superoxide dismutase activity (Brown, 1999). This flexible unstructured region of PrP (Donne *et al.*, 1997; Riek *et al.*, 1997) might be important for the physiological function of the prion protein and might be involved in the PrP^{c}/PrP^{Sc} conversion process. The internalization process of the prion protein is governed by metal binding to octarepeats (Sumudhu *et al.*, 2001). Very recently, the octarepeat region has been identified as an indirect interaction domain for the binding of the prion protein to its 37 kDa/67 kDa laminin receptor mediated by cell surface heparan sulfate proteoglycans (HSPGs), which act as co-factors/co-receptors for the PrP binding/internalization process (Hundt *et al.*, 2001). Biosensor and enzyme-linked immunosorbent assay methodologies identified, in addition to the octarepeat region of the prion protein (aa53-93), regions stretching from aa23-52 and aa110-128 of the prion protein as heparin and heparan sulfate binding sites (Warner *et al.*, 2002). Interestingly, the interaction of the octarepeat-spanning peptide (aa53-93) with heparin was enhanced by Cu(II) ions. A peptide encompassing the octarepeat region was able to inhibit the binding of full-length PrP to heparin, which suggests a direct role in heparin recognition within the intact prion protein (Warner *et al.*, 2002).

Deletion of the octarepeat region resulted in a weaker PrP/PrP interaction in the yeast two-hybrid system, suggesting that a second PrP/PrP interaction domain within the carboxy-terminal part of PrP may exist. Co-expression of PrP90-230 in both positions of the yeast two-hybrid system resulted in a direct interaction between both truncated PrP molecules, demonstrating that the carboxy-terminus of PrP (PrP90-230) represents a second PrP/PrP interaction domain. Since DTT affects PrP dimerization only marginally (Figure 1J), we conclude that intermolecular disulfide bridges do not, or only marginally, contribute to PrP dimer formation.

Influence of Additional Octarepeats in the PrP/PrP Interaction Processes

Additional octarepeats have been identified in patients suffering from familial CJD (Owen *et al.*, 1990, 1992; Goldfarb *et al.*, 1991, 1993; Campbell *et al.*, 1996). PrP encompassing nine additional octarepeats associated with familial CJD failed to undergo Cu^{2+}-mediated endocytosis, suggesting that neurodegeneration may arise from the ablation of internalization due to mutation of the octarepeats (Sumudhu *et al.*, 2001). Since these mutations affect the octarepeat region as one PrP/PrP interaction domain, we investigated whether these mutations may influence the PrP/PrP interaction behavior. Expression of mutated PrP encompassing two, five and nine additional octarepeats in the bait position *versus* wild-type PrP in the prey position of the yeast two-hybrid system slightly diminished the PrP/PrP interaction process. This situation mimics heterozygous CJD patients with the mutated *Prn*-p gene on one allele and the wild-type *Prn*-p on the other (Majtenyi *et al.*, 2000). Expression of mutated PrP with five and nine additional octarepeats in both positions of the yeast two-hybrid system resulted in total inhibition of the PrP/PrP interaction, reflecting to our knowledge a hypothetical homozygous CJD patient expressing this mutated PrP on both alleles of the *Prn*-p gene. Our results suggest that PrP/PrP interactions take place in all heterozygous CJD patients expressing additional octarepeats.

Transgenic mice expressing a mutant PrP encompassing nine additional octarepeat copies exhibit a slowly progressive neurological disorder characterized clinically by ataxia and neuropathologically by cerebellar atrophy and granule cell loss, gliosis, and PrP deposition that is most prominent in the cerebellum and hippocampus (Chiesa *et al.*, 1998). Moreover, these mice produce PrP that is more pK resistant than normal PrP^{c} (though it seems not to represent bona fide PrP^{Sc}), which accumu-

lates concomitant with massive apoptosis of granule cells in the cerebellum (Chiesa *et al.*, 2000). These features are more pronounced in homozygous [Tg(PG14$^{+/+}$)] than in heterozygous [Tg(PG14$^{+/-}$)] mice (Chiesa *et al.*, 2000). These data, together with the observation that additional octarepeats cause familial CJD in humans, demonstrate that the additional nine octarepeats indeed have a pathogenic effect. Very recently, we detected that recombinant huPrP encompassing nine additional octarepeats synthesized in the Semliki Forest Virus (SFV) system revealed proteinase K resistance (Gauczynski *et al.*, 2002), suggesting that this mutant prion protein might encompass endogenous infectivity. Non-dimerized PrP^{c} may not be able to fulfill its normal physiological function and be abnormally processed. The recent finding that nine additional octarepeats in PrP prevent the protein from copper-mediated endocytosis (Brown *et al.*, 1997) provides one explanation for the neurodegeneration observed in patients encompassing this mutation. Whether the wild-type prion protein appears monomeric or dimeric at the cell surface remains to be investigated.

Moreover, a free N-terminus might render the non-dimerized prion protein more prone to misfolding and probably to conversion into PrP^{Sc}. We hypothesize that the availability of the second carboxy-terminal PrP/PrP interaction domain is then required for PrP^{Sc} to convert more free PrP^{c} molecules, resulting in prion propagation. This requirement is fulfilled in heterozygous cases of familial CJD, were PrP/PrP interaction does still occur as shown in the yeast two-hybrid analysis (PrP+9OR *versus* wild-type PrP).

Hence, we speculate that PrP/PrP interaction *via* the octarepeat binding domain may be important for the normal physiological function of PrP and for the stabilization of PrP^{c}/PrP^{c} dimers. In the heterozygous case, PrP^{c}/PrP^{c} interaction is reduced but PrP^{c}/PrP^{Sc} interaction does still occur, most likely *via* the second PrP/PrP interaction domain (PrP90-230). We speculate that this binding domain might be important for the PrP^{c}/PrP^{Sc} conversion process, since PrP90-230 dimers have been identified within the PrP oligomerization/multimerization process analyzed by FCS (Post *et al.*, 1998; Jansen *et al.*, 2001).

Influence of TSE Relevant Mutations and Polymorphisms on the PrP/PrP Interaction Behavior

Defined mutations within the human *Prn*-p gene, such as the Leu102Pro mutation or the Asp178Asn mutation, lead to GSS and FFI, respectively (for a review see Lasmézas and Weiss, 2000). The polymorphism at position 129 Met/Val influences the susceptibility of humans toward FFI, fCJD and nvCJD. Interestingly enough, all patients suffering from nvCJD investigated so far are homozygous for methionine at this position (Zeidler, 1997; Hill *et al.*, 1999). All these mutations and the polymorphism either expressed in both positions or in bait *versus* wild-type PrP in the prey position of the yeast two-hybrid system did not affect the PrP/PrP interaction process. These findings are in good agreement with the assumption that only mutations such as E200K affecting helix 3 of the prion protein may interfere with PrP dimer formation *via* the structured region of PrP.

In summary, PrP/PrP interactions occur *via* the octarepeat region and the carboxy-terminal region from aa90 to 230 in the case of human PrP. From these findings we assume that interactions of the prion protein might occur in all patients suffering from GSS, FFI, fCJD and nvCJD and might be important for the PrP^{c}/PrP^{Sc} conversion process. Here, PrP/PrP interaction inhibitors may act as powerful tools in the therapy of TSEs. Also in the case of familial CJD caused by additional octarepeats, PrP/PrP interaction may still occur most likely *via* the PrP90-230 interaction domain. Blockage of this interaction may also result in an interference of the PrP^{Sc} replication process. In a homozygous case, in which PrP/PrP interaction might be completely blocked by five or nine additional octarepeats (here also the interaction *via* the second PrP binding domain PrP90-230 is impeded probably due to sterical reasons), the monomeric PrP might not be processed normally and may not fulfill its normal physiological functions, leading to the syndromes observed in transgenic mice expressing a mutated PrP with nine additional octarepeats. Such a PrP seems to aggregate in a more pK-resistant form, but prion replication has not been proved (Chiesa *et al.*, 1998, 2000). Very recently, we proved that the human PrP mutants D178N/M129 leading to FFI and huPrP+9OR leading to fCJD revealed proteinase K resistance, suggesting that they might be infectious and might lead to PrP replication (Gauczynski *et al.*, 2002).

Heterodimerization of Prion Proteins of Different Species

Investigating the interspecies interaction of prion proteins, we expressed prion proteins of different species in bait and prey positions of the yeast two-hybrid system. Bovine PrP interacted with human PrP, but hamster PrP failed to interact with bovine and human PrP (Table 1). These data are in fairly good accord with interspecies transmissions of prions. Indeed there is now convincing evidence from interspecies transmission studies in animals (Lasmézas *et al.*, 1996, 2001; Bruce *et al.*, 1997) and transgenic mice (Hill *et al.*, 1997; Scott *et al.*, 1999) that cattle BSE prions have been transmitted to humans (Table 1). Hamsters have not been successfully inoculated with bovine PrP (Bradley and Wilesmith, 1993), and Creutzfeldt-Jakob disease was only serially transmitted to Syrian hamsters *via* guinea pigs (Manuelidis *et al.*, 1978; Table 1). In the latter case, incubation times varied depending on the CJD strain used. Our yeast two-hybrid data further demonstrate an interaction between ovine PrP (AQ) and bovine or human PrP. There are no transmission data between sheep and humans (Table 1). However, the ovine scrapie agent has been transmitted to transgenic mice expressing bovine PrP (Scott *et al.*,

Table 1 Comparison of Interspecies Interactions in the Yeast Two-Hybrid System with Interconversion Studies Performed by the *in vitro* Conversion System[a] and Transmission Studies in Transgenic and Non-Transgenic Animals.

PrP species	PrP species	Interspecies interactions by the yeast two-hybrid system	Interconversions by *in vitro* conversion assays[a]	Interspecies transmission in transgenic mice	Interspecies transmission in animals
Human	Cattle	+	+[b]	+[c]	+[d]
Sheep	Cattle	+	+[e]	+[f]	+[g]
Sheep	Human	+	+[b]	n.d.	n.d.
Hamster	Human	–	n.d.	n.d.	*via* guinea pigs[h]
Hamster	Cattle	–	–[e]	n.d.	–[i]

'+' Interaction in the yeast two-hybrid system, interconversion by *in vitro* conversion assays, transmission in transgenic mice and non-transgenic animals.
'–' No interaction in the yeast two-hybrid system, no interconversion, no transmission in animals.
n.d.: not determined.
[a]According to Caughey *et al.* (1995).
[b]Raymond *et al.* (2000).
[c]Hill *et al.* (1997); Scott *et al.* (1999).
[d]Link between nvCJD and BSE demonstrated in macaques inoculated with PrP^{BSE} (Lasmézas *et al.*, 1996) and mice inoculated with PrP^{BSE}/PrP^{nvCJD} (Bruce *et al.*, 1997; Lasmezas *et al.*, 2001).
[e]Raymond *et al.* (1997).
[f]Scott *et al.* (2000).
[g]Cutlip *et al.* (1994); Clark *et al.* (1995).
[h]Manuelidis *et al.* (1978).
[i]Bradley and Wilesmith (1993).

2000). Cattle infected with the scrapie agent either intracerebrally (Cutlip *et al.*, 1994) or intramuscularly, subcutaneously or orally (Clark *et al.*, 1995) developed TSEs (Table 1). Although epidemiological data suggest that the ovine scrapie agent is hardly transmissible to humans, a sheep-human transmission that we suggest from our yeast two-hybrid data cannot be excluded. Employing the *in vitro* conversion system (Caughey *et al.*, 1995), inter-conversion studies have been performed that further confirm our interspecies interaction results obtained from the yeast two-hybrid system: both, bovine PrP^{BSE} and ovine PrP^{Sc} (AQ) converted human PrPsen, although only to a minimal extent, into a proteinase K-resistant form (Raymond *et al.*, 2000; Table 1). Sheep PrPsen (AQ) was converted by PrP^{BSE} into the pK resistant state (Raymond *et al.*, 1997; Table 1), whereas hamster PrPsen was not converted by bovine PrP^{BSE} (Raymond *et al.*, 1997; Table 1). In summary, our results of interspecies PrP interactions in the yeast two-hybrid system are in good harmony with transmission data obtained from transgenic and non-transgenic animals and with inter-conversion results obtained from the *in vitro* conversion assay. Therefore, we suggest that the yeast two-hybrid system acts as a fast pre-assay system to investigate species barriers in prion diseases.

Additional experiments including powerful PrP/PrP dimerization inhibitors might further clarify the role of PrP dimers in the replication mechanism of prions and the physiological function of PrP.

Materials and Methods

Recombinant Proteins Generated in the Baculovirus System

FLAG::haPrP23-231 has been generated as described previously (Rieger *et al.*, 1997). cDNAs encoding huPrP23-230, and bovPrP25-242 obtained from H. Kretzschmar (Munich, Germany) and M. Shinagawa (Obihiro, Japan), respectively, were generated by PCR and cloned into the transfer vector pFLAG-BAC (Rieger *et al.*, 1997) *via* *Bam*HI (5') and *Eco*RI (3'). The Hsp60 encoding cDNA was amplified from the vector pEt3a introducing *Bam*HI (5') and *Pst*I (3') restriction sites and cloned into the vector pFLAG-BAC (Rieger *et al.*, 1997). Recombinant viruses were generated by co-transfection of the transfer vectors with linearized viral DNA according to the manufacturer's instructions (Baculogold; Pharmingen). Recombinant FLAG::haPrP23-231, FLAG::huPrP23-230 and FLAG::bovPrP25-242 were expressed in baculovirus-infected Sf9 cells and purified to homogeneity as described previously for FLAG-tagged haPrP (Rieger *et al.*, 1997).

Recombinant pSFV Plasmid Construction

Construction of SFV1-LRP::FLAG was described by Gauczynski *et al.* (2001b). Construction of pSFV1-Hsp60::FLAG. The Hsp60 encoding cDNA was amplified by PCR from pEt3a introducing a *Bam*HI and a *Xma*I restriction site at the 5' and 3' ends. The 1755 bp fragment, which contains the Kozak sequence and AUG at the 5' end and a FLAG tag encoding sequence at the 3' end was cloned into the SFV expression plasmid pSFV1 (Liljestrom and Garoff, 1991) *via* *Bam*HI/*Xma*I restriction sites, resulting in pSFV1-Hsp60::FLAG. Construction of pSFV1-huPrP1-227FLAG228-253 and pSFV1-huPrP1-227HIS228-253. The insertion of a FLAG or a HIS tag encoding sequence between codons 227 and 228 of the human PrP sequence was done by PCR using the pSFV1-huPrP1-253 plasmid DNA as a template.

A 135 bp fragment (insertion of the FLAG encoding sequence) and a 129 bp fragment (insertion of the HIS encoding sequence), which both encode the carboxy-terminus of huPrP, were amplified, introducing a *Stu*I restriction site (endogenous site within codons 223–225) at the 5'-end, the tag-encoding sequence between codons 227 and 228, as well as a *Bam*HI site at the 3'-end. Both fragments were digested with *Stu*I and *Bam*HI and ligated *via* the *Stu*I restriction site to a 707 bp fragment encoding the amino terminal part of huPrP from pSFV1-huPrP1-253 digested with *Bam*HI and *Stu*I. The ligated DNA fragments were cloned into the expression plasmid pSFV1 *via* the *Bam*HI restriction sites resulting in pSFV1-huPrP1-227FLAG228-253 and pSFV1-huPrP1-227HIS228-253. The correct constructions of pSFV1-Hsp60::FLAG, pSFV1-huPrP1-227FLAG228-253 and pSFV1-huPrP1-227HIS228-253 was confirmed by dideoxysequencing. The plasmid DNA pSFV1-huPrP1-253 was described elsewhere (Krasemann *et al.*, 1996).

Preparation of SFV-mRNA *in vitro*

The recombinant plasmid DNAs pSFV1-huPrP1-227FLAG228-253, pSFV1-huPrP1-227HIS228-253, and pSFV1-LRP::FLAG were linearized with *Spe*I, the pSFV1-Hsp60::FLAG plasmid DNA was cut with *Sap*I (due to the internal *Spe*I restriction site within the encoding sequence). The linearized plasmid DNAs were purified by phenol-chloroform extraction followed by ethanol precipitation. Transcriptions were carried out in a total volume of 50 µl containing 1.5 µg linearized plasmid DNA, 10× SP6 transcription buffer (0.4 M Tris-HCl, pH 8.0 at 20 °C; 60 mM $MgCl_2$; 100 mM dithiothreitol; 20 mM spermidine), 1 mM of each ATP, CTP and UTP, 500 µM of GTP, 1 mM of $m^7G(5')ppp(5')G$, 50 units of RNasin and 50 units of SP6 RNA polymerase and incubated for 2 h at 37 °C. The correct length of the transcripts was proven by agarose gel electrophoresis. RNA was stored at –20 °C.

Mammalian Cell Culture, Transfection and Co-Transfection Studies with the Semliki Forest Virus (SFV) System

Baby hamster kidney cells (BHK-21 C13; ATCC CCL 10) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5% CO_2. Transfection and co-transfection were carried out by electroporation, where RNA was added directly from the *in vitro* transcription reaction to the BHK cells. BHK cells with a confluency of ~80% were rinsed with PBS (without $MgCl_2$ and $CaCl_2$), trypsinized, washed again and finally re-suspended in PBS to a density of 10^7 cells/ml. The total amount of transcribed RNA was mixed with 0.8 ml cells and the mixture transferred to a 0.4 cm cuvette. For co-transfection, both RNAs were added in proportion 1:1 to the cells. Electroporation was carried out at room temperature by two consecutive pulses at 850 V/25 µF using a Bio-Rad Gene Pulser. The time constant after each pulse should be 0.4 to 0.5. The total volume of the electroporated cells (8×10^6) was plated on 10 cm dishes containing 15 ml of complete growth medium. The cells were incubated at 37 °C with 5% CO_2 for 48 h.

Pull-Down Assays

BHK cells co-expressing huPrP-HIS and FLAG-tagged proteins such as huPrP::FLAG, LRP::FLAG, Hsp60::FLAG (SFV-system) were harvested 48 h post transfection, washed once with PBS and then lysed in PBS supplemented with 0.1% Triton-X-100 at 4 °C. The crude lysates were obtained by centrifugation at 20200 *g* at 4 °C for 15 min and purified by the batch method using a Chelating Sepharose Fast Flow gel (Pharmacia Biotech) charged with nickel. The histidine-tagged protein (huPrP-HIS) was bound overnight by rotating at 4 °C, washed four times with PBS and eluted overnight by competition with PBS containing 500 mM imidazole at 4 °C. In order to investigate the interaction between huPrP-HIS and the co-expressed FLAG-tagged proteins (mentioned above) the eluates were analyzed by Western blotting using the monoclonal anti-FLAG antibody M2 (Sigma).

Yeast Two-Hybrid Analysis

Construction of plasmid pSH2-1-GST::huPrP23-230 was described previously (Rieger *et al.*, 1997). The cloning procedure of all other human PrP constructs into the vector pSH2-1 was analogous. The GST::huPrP23-230 encoding cDNA was excised from pSH2-1-GST::huPrP23-230 and subcloned into pJG4-5 *via Eco*RI and *Sal*I. All other constructs were cloned into the vector pJG4-5 in the same way. The construct pSH2-1-GST::huPrPΔGP lacking the octarepeat region aa 51-91 was generated by Kunkel mutagenesis (Kunkel, 1985). The constructs pSH2-1-GST::huPrPGP (aa52-93), pSH2-1-GST::huPrP23-50 and pSH2-1-GST::huPrP90-230 were amplified by PCR using oligodesoxyribonucleotides coding for the different PrP-sequences flanked by *Bam*HI (5') and *Sal*I (3') restriction sites. The fragments were cloned *via Bam*HI and *Sal*I restriction sites into the vector pSH2-1. The construct pSH2-1-GST::huPrP23-230+9OR was subcloned from the vector pSFV1-huPrP+9OR (gift from Dr. S. Krasemann). The constructs pSH2-1-GST::huPrP23-230+2OR and pSH2-1-GST::huPrP23-230+5OR were generated by *Bst*XI restriction of the construct with additional 9 octarepeats and ligation of the restriction products with different lengths. This resulted in the insertion of 2 and 5 additional octarepeats. The constructs pSH2-1-GST::huPrP23-230P102L and pSH2-1-GST::huPrPM129V were cloned by Kunkel mutagenesis. The construct pSH2-1-GST::huPrPD178N (FFI) was subcloned from the plasmid pSFV1-huPrPD178N (FFI) (Krasemann *et al.*, 1996), which was a generous gift from Dr. S. Krasemann. The construct pSH2-1-GST::bovPrP25-242 was subcloned from the plasmid pSFV1-bov PrP *via Bam*HI (Krasemann *et al.*, 1996) and *Sal*I. The construct pSH2-1-GST::haPrP23-231 was subcloned from the plasmid pGEX-2T::haPrP23-231 (Weiss *et al.*, 1995) *via Bam*HI and *Sal*I. The construct pSH2-1-GST::shPrP25-234 (A/Q) was subcloned from ovine DNA (generous gift from W. Goldmann) *via Bam*HI and *Sal*I. All PrP constructs were confirmed by sequencing. The different bait plasmids, the prey plasmid pJG4-5-LRP and the reporter plasmid pSH18-34 (*lac*Z) were co-transformed into EGY48 cells and transformants were tested in β-galactosidase assays.

Proteinase K Digestion Assay

One hundred ng each of recombinant huPrP, bovPrP and haPrP synthesized in *E.coli* (Prionics) and 100 ng each of FLAG::huPrP23-230, FLAG::bovPrP25-242 and FLAG::haPrP23-231 synthesized in the baculovirus system purified by anti-FLAG antibody chromatography have been digested with a final concentration of 0, 1, 4, 8 and 20 µg/ml proteinase K in 50 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$. After 1 h at 37 °C, reactions were terminated with 1 mM pefabloc and analyzed by Western blotting employing the JB007 antibody.

SDS-PAGE, Native PA Gel Electrophoresis and Western Blotting

Proteins were analyzed by SDS-PAGE under reducing conditions (in the presence of 5 mM β-mercaptoethanol) followed by

silver staining of the gel or by Western blotting. Detection was performed by chemiluminescence. For analysis under native conditions, proteins were separated on native polyacrylamide gels (12% PAA bottom, 8% PAA middle and 6% PAA top). PAA was diluted in 1× TBE buffer. Protein samples were diluted in 40% glycine (diluted in water). Electrophoresis was performed at 25 mA for 1 h. Protein bands were electroblotted onto nitrocellulose and detection was performed employing the 3B5 antibody.

Size Exclusion Chromatography (SEC)

The Superose 12 PC 3.2/30 column (Amersham Pharmacia) was calibrated with the LMW calibration kit using the buffer 20 mM HEPES pH 7.4. 2.5 µg (25 µl) each of the homogeneous FLAG-tagged PrP from human, bovine and cattle expressed in the baculovirus system were loaded. The proteins were eluted with the same buffer at a flow rate of 30 µl/min and detected with a UV-M II monitor at 280 nm. For denaturation FLAG::huPrP23-230 was incubated with 100 mM DTT, 95 °C for 15 minutes.

Antibodies and Proteins

The monoclonal anti-PrP antibody 3B5 was kindly provided by G. Hunsmann, Göttingen, Germany, the polyclonal antibody JB007 was a kind gift of Corinne Ida Lasmézas, Fontenay-aux-Roses, France. Anti-FLAG antibody M2 was purchased from Sigma. Secondary anti-mouse IgG-POD conjugated was provided by Sigma. Monomeric recombinant prion protein from man, cattle and hamster were obtained from Prionics, Switzerland.

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References

Alper, T., Cramp, W.A., Haig, D.A. and Clarke, M.C. (1967). Does the agent of scrapie replicate without nucleic acid? Nature *214*, 764 – 766.

Bowman, A.B., Kamal, A., Ritchings, B.W., Philp, A.V., McGrail, M., Gindhart, J.G. and Goldstein, L.S. (2000). Kinesin-dependent axonal transport is mediated by the sunday driver (SYD) protein. Cell *103*, 583 – 594.

Bradley, R. and Wilesmith, J.W. (1993). Epidemiology and control of bovine spongiform encephalopathy (BSE). Br. Med. Bull. *49*, 932 – 959.

Brown, D.R. (1999). Comment on: Neurotoxicity of prion peptide 106 – 126 not confirmed. FEBS Lett. *460*, 65 – 68.

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von, B.A., Schulz, S.W., Giese, A., Westaway, D. and Kretzschmar, H. (1997). The cellular prion protein binds copper *in vivo*. Nature *390*, 684 – 687.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C. *et al.* (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature *389*, 498 – 501.

Campbell, T.A., Palmer, M.S., Will, R.G., Gibb, W.R., Luthert, P.J. and Collinge, J. (1996). A prion disease with a novel 96-base pair insertional mutation in the prion protein gene. Neurology *46*, 761 – 766.

Caughey, B., Kocisko, D.A., Raymond, G.J. and Lansbury Jr., P.T., (1995). Aggregates of scrapie-associated prion protein induce the cell-free conversion of protease-sensitive prion protein to the protease-resistant state. Chem. Biol. *2*, 807 – 817.

Chiesa, R., Piccardo, P., Ghetti, B. and Harris, D.A. (1998). Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. Neuron *21*, 1339 – 1351.

Chiesa, R., Drisaldi, B., Quaglio, E., Migheli, A., Piccardo, P., Ghetti, B. and Harris, D.A. (2000). Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation. Proc. Natl. Acad. Sci. USA *97*, 5574 – 5579.

Clark, W.W., Hourrigan, J.L. and Hadlow, W.J. (1995). Encephalopathy in cattle experimentally infected with the scrapie agent. Am. J. Vet. Res. *56*, 606 – 612.

Cutlip, R.C., Miller, J.M., Race, R.E., Jenny, A.L., Katz, J.B., Lehmkuhl, H.D., DeBey, B.M. and Robinson, M.M. (1994). Intracerebral transmission of scrapie to cattle. J. Infect. Dis. *169*, 814 – 820.

Donne, D.G., Viles, J.H., Groth, D., Mehlhorn, I., James, T.L., Cohen, F.E., Prusiner, S.B., Wright, P.E. and Dyson, H.J. (1997). Structure of the recombinant full-length hamster prion protein PrP(29 – 231): the N terminus is highly flexible. Proc. Natl. Acad. Sci. USA *94*, 13452 – 13457.

Edenhofer, F., Rieger, R., Famulok, M., Wendler, W., Weiss, S. and Winnacker, E.L. (1996). Prion protein PrPc interacts with molecular chaperones of the Hsp60 family. J. Virol. *70*, 4724 – 4728.

Gauczynski, S., Hundt, C., Leucht, C. and Weiss, S. (2001a). Interaction of prion proteins with cell surface receptors, molecular chaperones, and other molecules. Adv. Prot. Chem. *57*, 229 – 272.

Gauczynski, S., Peyrin, J.-M., Haïk, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J.-P., Dormont, D., Lasmézas, C.I. and Weiss, S. (2001b). The 37 kDa/67 kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J. *20*, 5868 – 5875.

Gauczynski, S., Krasemann, S., Bodemer, W. and Weiss, S. (2002). Recombinant human prion protein mutants huPrPD178N/M129 (FFI) and huPrP+9OR (fCJD) reveal proteinase K resistance. J. Cell Sci. *115*, 4025 – 4036.

Goldfarb, L.G., Brown, P., McCombie, W.R., Goldgaber, D., Swergold, G.D., Wills, P.R., Cervenakova, L., Baron, H., Gibbs, C.J. and Gajdusek, D.C. (1991). Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene. Proc. Natl. Acad. Sci. USA *88*, 10926 – 10930.

Goldfarb, L.G., Brown, P., Little, B.W., Cervenakova, L., Kenney, K., Gibbs Jr., C.J., and Gajdusek, D.C. (1993). A new (two-repeat) octapeptide coding insert mutation in Creutzfeldt-Jakob disease. Neurology *43*, 2392 – 2394.

Goldgaber, D., Goldfarb, L.G., Brown, P., Asher, D.M., Brown, W.T., Lin, S., Teener, J.W., Feinstone, S.M., Rubenstein, R., Kascsak, R.J. *et al.* (1989). Mutations in familial Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker's syndrome. Exp. Neurol. *106*, 204 – 206.

Griffith, J.S. (1967). Self-replication and Scrapie. Nature *215*, 1043 – 1044.

Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993). Cdi1,

a human G1 and S phase protein phosphatase that associates with Cdk2. Cell *75*, 791 – 803.

Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J. and Lantos, P. (1997). The same prion strain causes vCJD and BSE. Nature *389*, 448 – 450.

Hill, A.F., Butterworth, R.J., Joiner, S., Jackson, G., Rosser, M. N., Thomas, D. J., Frosh, A., Tolley, N., Bell, J.E., Spencer, M. *et al.* (1999). Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. Lancet *353*, 183 – 189.

Hundt, C., Peyrin, J.-M., Haïk, S., Gauczynski, S., Leucht, C., Riley, M.-L., Rieger, R., Deslys, J.-P., Dormont, D., Lasmézas, C.I. and Weiss, S. (2001). Identification of interaction domains of the prion protein with its 37 kDa/67 kDa laminin receptor. EMBO J. *20*, 5876 – 5886.

Jansen, K., Schafer, O., Birkmann, E., Post, K., Serban, H., Prusiner, S.B. and Riesner, D. (2001). Structural intermediates in the putative pathway from the cellular prion protein to the pathogenic form. Biol. Chem. *382*, 683 – 691.

Kaneko, K., Zulianello, L., Scott, M., Cooper, C.M., Wallace, A.C., James, T.L., Cohen, F.E. and Prusiner, S.B. (1997). Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. Proc. Natl. Acad. Sci. USA *94*, 10069 – 10074.

Knaus, K.J., Morillas, M., Swietnicki, W., Malone, M., Surewicz, W.K. and Yee, V.C. (2001). Crystal structure of the human prion protein reveals a mechanism for oligomerization. Nature Struct. Biol. *8*, 770 – 774.

Krasemann, S., Groschup, M.H., Harmeyer, S., Hunsmann, G. and Bodemer, W. (1996). Generation of monoclonal antibodies against human prion proteins in $prp^{0/0}$ mice. Mol. Med. *2*, 725 – 734.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA *82*, 488 – 492.

Lansbury, P.T.J. and Caughey, B. (1995). The chemistry of scrapie infection: implications of the 'ice 9' metaphor. Chem. Biol. *2*, 1 – 5.

Lasmézas, C.I., Deslys, J.P., Demamay, R., Adjou, K.T., Lamoury, F., Dormont, D., Robain, O., Ironside, J. and Hauw, J.J. (1996). BSE transmission to macaques. Nature *381*, 743 – 744.

Lasmézas, C.I. and Weiss, S. (2000). Molecular Biology of Prion Diseases. In: Microbial Foodborne Diseases. Mechanisms of Pathogenicity and Toxin Synthesis, J.W. Cary, J.E. Linz and D. Bhatnagar, eds. (Lancaster, USA: Technomic Publishing), pp. 495 – 537.

Lasmezas, C.I., Fournier, J.G., Nouvel, V., Boe, H., Marce, D., Lamoury, F., Kopp, N., Hauw, J.J., Ironside, J., Bruce, M., Dormont, D. and Deslys, J.P. (2001). Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: implications for human health. Proc. Natl. Acad. Sci. USA *98*, 4142 – 4147.

Leucht, C. and Weiss, S. (2002). Der Prion Protein Rezeptor. Nova Acta Leopoldina *87*, 39 – 54.

Leucht, C., Simoneau, S., Rey, C., Vana, K., Rieger, R., Lasmézas, C.I. and Weiss, S. (2003). The 37 kDa/67 kDa laminin receptor is required for PrP^{SC} propagation in scrapie infected neuronal cells [correction published in EMBO Rep. *4* (2003), p. 439]. EMBO Rep. *4*, 290 – 295.

Liljestrom, P. and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology *9*, 1356 – 1361.

Lopez, L., Urzainqui, A., Dominguez, E. and Garcia, J.A. (2001). Identification of an N-terminal domain of the plum pox potyvirus CI RNA helicase involved in self-interaction in a yeast two-hybrid system. J. Gen. Virol. *82*, 677 – 686.

Majtenyi, C., Brown, P., Cervenakova, L., Goldfarb, L.G. and Tateishi, J. (2000). A three-sister-sibship of Gerstmann-Straussler-Scheinker disease with a CJD phenotype. Neurology *54*, 2133 – 2137.

Manuelidis, E.E., Gorgacz, E.J. and Manuelidis, L. (1978). Interspecies transmission of Creutzfeldt-Jakob disease to Syrian hamsters with reference to clinical syndromes and strains of agent. Proc. Natl. Acad. Sci. USA *75*, 3432 – 3436.

Meier, P., Genoud, N., Prinz, M., Maissen, M., Rülicke, T., Zurbriggen, A., Raeber, A.J., and Aguzzi, A. (2003). Soluble dimeric prion protein binds PrP^{Sc} *in vivo* and antagonizes prion disease. Cell *113*, 49 – 60.

Meyer, R.K., Lustig, A., Oesch, B., Fatzer, R., Zurbriggen, A. and Vandevelde, M. (2000). A monomer-dimer equilibrium of a cellular prion protein (PrPc) not observed with recombinant PrP. J. Biol. Chem. *275*, 38081 – 38087.

Owen, F., Poulter, M., Shah, T., Collinge, J., Lofthouse, R., Baker, H., Ridley, R., McVey, J. and Crow, T.J. (1990). An in-frame insertion in the prion protein gene in familial Creutzfeldt-Jakob disease. Mol. Brain Res. *7*, 273 – 276.

Owen, F., Poulter, M., Collinge, J., Leach, M., Lofthouse, R., Crow, T.J. and Harding, A.E. (1992). A dementing illness associated with a novel insertion in the prion protein gene. Mol. Brain Res. *13*, 155 – 157.

Post, K., Pitschke, M., Schafer, O., Wille, H., Appel, T.R., Kirsch, D., Mehlhorn, I., Serban, H., Prusiner, S.B. and Riesner, D. (1998). Rapid acquisition of β-sheet structure in the prion protein prior to multimer formation. Biol. Chem. *379*, 1307 – 1317.

Priola, S.A. and Chesebro, B. (1998). Abnormal properties of prion protein with insertional mutations in different cell types. J. Biol. Chem. *273*, 11980 – 11985.

Priola, S.A., Caughey, B., Wehrly, K. and Chesebro, B. (1995). A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. J. Biol. Chem. *270*, 3299 – 3305.

Prusiner, S.B. (1982). Novel proteinaceous infectious particles cause Scrapie. Science *216*, 136 – 144.

Prusiner, S.B., Scott, M.R., DeArmond, S.J. and Cohen, F.E. (1998). Prion protein biology. Cell *93*, 337 – 348.

Raymond, G.J., Hope, J., Kocisko, D.A., Priola, S.A., Raymond, L.D., Bossers, A., Ironside, J., Will, R.G., Chen, S.G., Petersen, R.B. *et al.* (1997). Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. Nature *388*, 285 – 288.

Raymond, G.J., Bossers, A., Raymond, L.D., O'Rourke, K.I., McHolland, L.E., Bryant, P.K., 3rd, Miller, M.W., Williams, E.S., Smits, M. and Caughey, B. (2000). Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. EMBO J. *19*, 4425 – 4430.

Rieger, R., Edenhofer, F., Lasmezas, C.I. and Weiss, S. (1997). The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. Nature Med. *3*, 1383 – 1388.

Rieger, R., Lasmezas, C.I. and Weiss, S. (1999). Role of the 37 kDa laminin receptor precursor in the life cycle of prions. Transfus. Clin. Biol. *6*, 7 – 16.

Riek, R., Hornemann, S., Wider, G., Glockshuber, R. and Wuthrich, K. (1997). NMR characterization of the full-length recombinant murine prion protein, mPrP(23-231). FEBS Lett. *413*, 282 – 288.

Riley, M.-L., Leucht, C., Gauczynski, S., Hundt, C., Brecelj, M., Dodson, G. and Weiss, S. (2002). High-level expression and characterization of a glycosylated covalently linked dimer of the prion protein. Protein Eng. *15*, 529 – 537.

Scott, M.R., Supattapone, S., Nguyen, H.O., DeArmond, S.J. and Prusiner, S.B. (2000). Transgenic models of prion disease. Arch. Virol. (Suppl.) *16*, 113 – 124.

Scott, M.R., Will, R., Ironside, J., Nguyen, H.O., Tremblay, P., DeArmond, S.J. and Prusiner, S.B. (1999). Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc. Natl. Acad. Sci. USA *96*, 15137–15142.

Simoneau, S., Haïk, S., Leucht, C., Dormont, D., Deslys, J.-P., Weiss, S. and Lasmézas, C. (2003). Different isoforms of the non-integrin laminin receptor are present in mouse brain and bind PrP. Biol. Chem. *384*, 243–246.

Sumudhu, W., Perera, S. and Hooper. (2001). Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region. Curr. Biol. *11*, 519–522.

Tateishi, J., Brown, P., Kitamoto, T., Hoque, Z.M., Roos, R., Wollman, R., Cervenakova, L. and Gajdusek, D.C. (1995). First experimental transmission of fatal familial insomnia. Nature *376*, 434–435.

Warner, R.G., Hundt, C., Weiss, S. and Turnbull, J.E. (2002). Identification of the heparan sulfate binding sites in the cellular prion protein. J. Biol. Chem. *277*, 18421–18430.

Warwicker, J. (1997). Species barriers in a model for specific prion protein dimerization. Biochem. Biophys. Res. Commun. *232*, 508–512.

Warwicker, J. and Gane, P.J. (1996). A model for prion protein dimerisation based on α-helical packing. Biochem. Biophys. Res. Commun. *226*, 777–782.

Weiss, S., Famulok, M., Edenhofer, F., Wang, Y.H., Jones, I.M., Groschup, M. and Winnacker, E.L. (1995). Overexpression of active Syrian golden hamster prion protein PrPc as a glutathione S-transferase fusion in heterologous systems. J. Virol. *69*, 4776–4783.

Weissmann, C. and Aguzzi, A. (1997). Bovine spongiform encephalopathy and early onset variant Creutzfeldt-Jakob disease. Curr. Opin. Neurobiol. *7*, 695–700.

Zeidler, M., Stewart, G., Cousens, S.N., Estibeiro, K. and Will, R.G. (1997). Codon 129 genotype and new variant CJD. Lancet *350*, 668.

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