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Interaction studies of the cellular prion protein

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Prof. Dr. Ernst-Ludwig Winnacker betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Christoph Hundt

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Meinen Eltern

Summary

Prion diseases are rare but fatal neurodegenerative diseases which occur both in humans and mammals caused by the prion protein (PrP) which is well conserved among the species. In this thesis the biochemical properties and the function of prion protein were investiagted using different methods.

The oligomerisation state of the prion protein analysed by size exclusion chromatography revealed that the prion protein is dimeric under native conditions. This was proven in the yeast two-hybrid system followed by the identification of two interaction domains. The influence of mutations and polymorphisms within the prion gene was investigated in the yeast two-hybrid system. This method is also an useful tool for the investigation of the species barrier.

To determine the role of dimeric prion proteins on the scrapie prion protein formation a covalently-linked PrP dimer was constructed and expressed in yeast *Pichia pastoris*. The protein was expressed as a glycosylated, proteinase K sensitive protein which is transported to the plasma membrane of yeast cells.

Recently, the 37-kDa/67-kDa laminin receptor LRP/LR was identified as the receptor for cellular PrP. Besides the *in vitro* interaction of PrP and LRP the binding domains on PrP and LRP were mapped in the yeast two-hybrid system. In addition, cell binding assays revealed a second HSPG-dependent binding domain leading to a comprehensive model of the PrP/LRP complex on the cell surface.

The binding of heparan sulfates to the prion protein was investigated and binding domains located on the N-terminus of the prion protein were characterized using biosensor and ELISA methodology.

The prion-like protein Doppel (Dpl) shows neither an interaction with PrP nor with LRP, the receptor for the cellular prion protein, in the yeast two-hybrid system, but is well expressed in yeast cells.

The tyrosine kinase Fyn, activated by PrP in neuronal cells, interact directly with PrP in the yeast two-hybrid system whereas LRP failed to interact with Fyn.

Chapter I

Introduction

1. Transmissible Spongiform Encephalopathies (TSEs)

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases described for human and animals. TSEs include Creutzfeldt-Jakob disease (CJD) (Creutzfeldt, 1920), Gerstmann-Sträussler-Scheinker syndrome (GSS) (Gerstmann, 1928), Kuru (Zigas, 1970) and fatal familial insomnia (FFI) (Lugaresi *et al*., 1986) in humans, scrapie in sheep (Hadlow *et al*., 1982) and BSE in cattle (Wells *et al*., 1987). In addition, TSEs are known for felines (Pearson *et al*., 1992) (FSE), goat (Pattison, 1965), elk and deer (Guiroy *et al*., 1991), monkeys (Gibbs and Gajdusek, 1972) and other animals. In every species TSEs represents a fatal disease leading to death. The clinical presentation of the disease includes behavioural changes, motoric troubles, progressive dementia and ataxia. The main neuropathologic features of the disease is a typical lesion of the CNS along with neuronal loss, spongiosis, gliosis and astrogliosis (Lantos *et al*., 1997). Signs of a conventional viral infection were never detectable in the CNS. This leads to the conclusion that the causing agent of the TSEs is a new class of infectious agents termed prions (abbreviation for proteinaceous infectious particle). Prions are proteins devoid of any nucleic acid (for review see Lasmézas and Weiss, 2000; Prusiner, 1982) and are resistant against proteinase digestion and chemical and physical treatment (Bessen and Marsh, 1992). The lack of any nucleic acid in purified preparations of prions (Kellings *et al*., 1992) makes it necessary to postulate a new mechanism for the replication of the prion protein. The most accepted one is the "protein-only“ hypothesis proposed by S. B. Prusiner (Prusiner, 1982; Prusiner, 1991).

1.1. BSE epidemiology

The first suspected BSE cases in Europe were recognised in the United Kingdom in 1985 and the first case was diagnosed in 1986 (Wells *et al*., 1987). The number of cases arose and reached its maximum in 1992 with over 37000 cases (Fig.1). Two hypotheses about the origin of the BSE epidemic exist (Fraser, 2000). According to the sheep origin hypothesis, the agent causing the disease was transmitted from sheep to cattle by feeding of meat and bone meal prepared from sheeps suffering from scrapie. Scrapie is a well known disease in sheep since the 18^{th} century (Hadlow *et al*., 1982). Meat and bone meal was inactivated by sterilization with temperatures more than 130°C presumably inactivating the pathogenic prion protein. In the early 80s the sterilization temperature was reduced to about 110°C which was insufficient to inactivate the scrapie agent, allowing the agent to cross the species barrier between sheep and cattle resulting in the BSE epidemic. One and a half year after the first histopathologic

confirmation of BSE in 1986 the „feed ban“ was introduced which prohibited the feeding of meat and bone meal to animals followed by the specified offal ban in 1989. The BSE cases increased until 1992 due to the long incubation period of the disease (3-7 years in case of cattle) (Wilesmith *et al*., 1991). In consequence of the feed bans a decrease of the number of BSE cases was observed after 1992. The bovine origin hypothesis states that the infectious agent originates from cattle which might develop the prion disease spontaneously (with an expected incidence rate of 1/1.000.000 cattle).

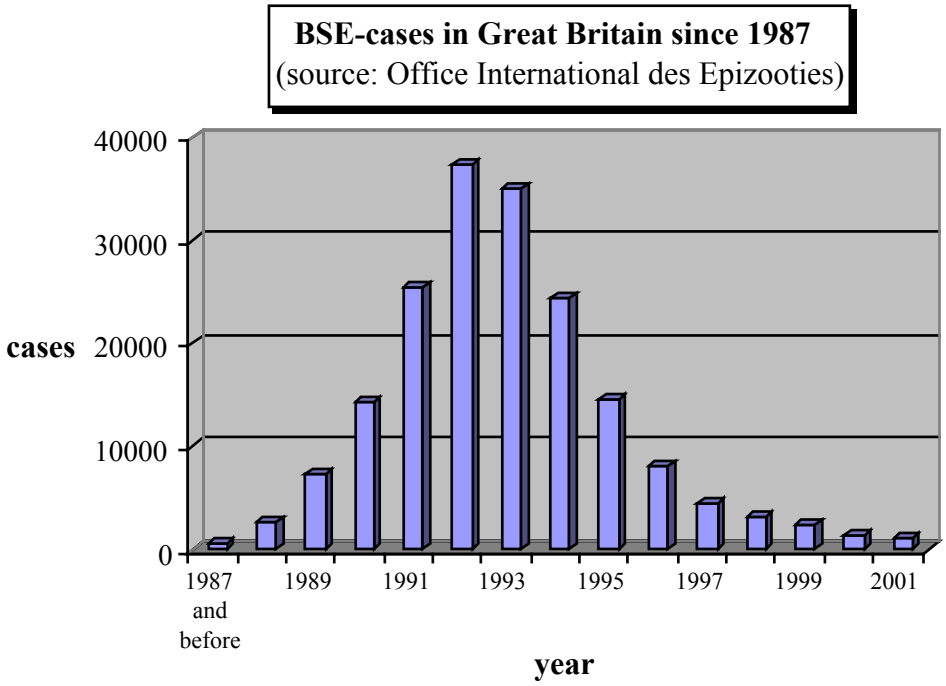

Fig. 1: Confirmed cases of BSE in the United Kingdom plotted per year of clinical onset. Data were provided by the Office International des Epizooties (www.oie.int).

BSE is not only a problem of the United Kingdom. It was spread to a series of European countries (table1). The appearence of BSE outside the UK is probably related with the import and feeding of meat and bone meal contaminated with prions in these countries. E.g: strain typing of a Swiss BSE case revealed the same results to that of those tested in the UK (Vandevelde *et al*., 1992) which suggests the same origin of the disease. The expected number of BSE cases in countries of continental Europe might not reach the level of the UK, but is expected to further increase.

Table 1:Number of confirmed BSE cases in Europe (source: www.ourworld-top.cs.com, 05. January 2002)

Country	Number of BSE cases since 1987
United Kingdom	181720
Ireland	843
Portugal	597
France	515
Switzerland	399
Germany	138
Spain	84
Belgium	65
Italy	48
Netherland	28
Denmark	8
Slovakia	5
Lichtenstein	2
Czech. Republic	2
Greece	1
Austria	1
Finland	1
Luxembourg	1
Slovenia	1

Further cases: Japan: 3, Oman:2, Falkland Islands. 1 and Canada 1

1.2. Transmission studies involving the BSE agent

Many experiments have demonstrated that the BSE agent is able to cross the species barrier. Inoculation of the prion agent into a new host species might prolonge the incubation period and may reduce the transmission rates. To study the transmission of prions from one species to another four methods have been established: intracerebral, intraperitoneal and oral inoculation of host species with prions (Race *et al*., 2001), usage of transgenic mice (Telling, 2000), *in vitro* conversion assays (Caughey *et al*., 1995) and the yeast two-hybrid system (see chapter III).

By oral infection the BSE agent was directly transmitted to sheep (Bradley and Wilesmith, 1993), to goats (Bradley and Wilesmith, 1993) and to mice (Barlow and Middleton, 1990), but not to chicken and pigs (Bradley and Wilesmith, 1993). Interestingly the scrapie agent from sheep has also been transmitted to cows (Cutlip *et al*., 1994) suggesting that the BSE epidemic was indeed induced by the scrapie agent.

For ethical reasons the direct route of inoculation is sometimes impossible i.e. in case od humans and cattle. Thus the usage of transgenic mice represents the appropriate method. In some experiments the endogenous mouse prion gene *Prn*-p has been knocked out and the *Prn*-p gene of a foreign species has been introduced. Among many transmission experiments the transmission of hamster prions to mice transgenic for hamster PrP ($haPrP^{+/+}/moPrP^{-/-}$) lead to the disease, whereas the transmission to wild type mice failed (Scott *et al*., 1989) indicating a species barrier for the transmission of prions to foreign mammals.

One crucial step in prion diseases is the conversion of the cellular PrP into the pathogenic isoform. According to the protein-only hypothesis the conversion proceeds by interaction of the infectious prion protein (PrP^{Sc}) with the normal prion protein (PrP^{c}) (Prusiner, 1982). In *in vitro* conversion assays the conversion of cellular proteinase K sensitive PrP (PrPsen) of human and sheep to the proteinase K resistant isoform (PrPres) driven by BSE prions was proven (Raymond *et al*., 2000). In these experiments, however, infectious material has never been generated.

1.3. New variant CJD

Sporadic Creutzfeldt-Jakob disease (sCJD) is a very rare neurodegenerative disease in humans which was initially described in the 1920s (Creutzfeldt, 1920). Today the incidence rate of CJD is about one case in 10^6 individuals (Alperovitch *et al*., 1994). There are three reasons for the development of CJD: 85% of the cases are sporadic without any known origin, 15% are familial due to a mutation within the *Prn*-p gene and some cases have an iatrogenic background where the infection occurs via contact with contaminated surgical instruments or the application of human growth hormon (HGH) (Buchanan *et al*., 1991). The progression of CJD is very rapid and leads to death about one year after the onset of the disease because of spongiform degeneration and astrogliosis. The age of the patients ranges between 60 and 74 years.

A novel form of CJD was recognised in the UK in 1996 termed new variant CJD (nvCJD) (Will *et al*., 1996) and epidemiological studies implied a link with BSE. Until 07 January 2002 104 confirmed cases and 9 probable cases were reported in the UK (source: The Creutzfeldt-Jakob Disease Surveillance Unit, Edinbourgh, UK) and 4 cases were found in France (Lasmézas, C.I. personal communication). The main difference between nvCJD and the sporadic or familial CJD is the young age (mean age is 29 years) of the nvCJD patients. In addition, investigation of brains of nvCJD patients revealed a special form of amyloid plaques (florid-type plaques) due to their daisy-like appearance (Ironside and Bell, 1997). The most

compelling evidence that nvCJD is caused by BSE prions came from studies with transgenic mice (Scott *et al*., 1999). Transgenic mice expressing bovine PrP (bovPrP$^{+/+}$/moPrP$^{-/-}$) propagate BSE prions indicating that there is no species barrier between cattle and these transgenic mice. These mice were also highly susceptible to nvCJD leading to the same features of the disease regarding incubation time, neuropathology and PrP^{Sc} deposition whether the inoculate originates from cattle or a nvCJD patient. Wild type mice (C57BL6) developed the disease after similar incubation time when inoculated with the nvCJD or BSE agent (Bruce *et al*., 1997). Further macaques inoculated with the BSE agent showed the same floride-type plaques as nvCJD patients (Lasmézas *et al*., 1996). All patients analysed so far were homozygous for methionine at codon 129 of the *Prn*-p gene, which is the genotype of 38% of the white population (Jackson and Collinge, 2001) and all cattle analysed so far were homozygous for methionine at the corresponding bovine codon (Goldmann *et al*., 1991). Homozygocity at position 129 for methionine seems to be a genetic predisposition to develop nvCJD. Recently, the human leukocyte antigen (HLA) class-II type DQ7 was identified as a diagnostic marker for nvCJD because of its reduced frequency in the nvCJD cases compared to classical CJD cases (Jackson *et al.*, 2001).

The infection of humans with BSE implies an oral route of infection. About 50000 BSE-infected cattle are supposed to have entered the human food chain (Anderson *et al*., 1996). However, time humans have been exposed to the infectious agent, source of the infectious BSE agent and duration of the exposition to the agent of the nvCJD cases were not comprehensible. Extrapolation of data from mice models to humans revealed that the mean incubation time for the development of nvCJD caused by BSE might be about 30 years. However, the incubation period for sporadic CJD is estimated to be about 12 years (Brown *et al*., 1992).

The BSE agent replicates within the lymphoreticular system when coming from the gastrointestinal area after oral infection on its way to the brain (Hill *et al*., 1999). It is impossible to predict at the moment whether nvCJD in humans will be epidemiologically comparable to BSE in cattle.

1.4. Familial forms of human TSE

The *Prn*-p gene encoding the prion protein is a single-copy gene, which is located on chromosome 20 (Sparkes *et al*., 1986) and consists of three exons (Kretzschmar *et al*., 1986). Among different species the sequence of the prion protein is highly conserved (Schätzl *et al*., 1995) indicating the importance of PrP^c for the organisms.

Within the *Prn*-p gene point mutations, a deletion and insertions are reported which are associated with the development of familial human TSEs (Fig.2). Three different types of familial human TSE are known depending on the appearance of the disease: sporadic Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). The CJD phenotype is characterized by rapid progressing dementia and periodic synchronized discharges in the electroencephalogram (EEG) (Bell and Ironside, 1993). The following mutations cause CJD: M178V (together with 129V), V180I, E200K, R208H, V210, M232R, deletion of one octarepeat and insertion of 1, 2, 4, 5, 6, 7, 8 and 9 octarepeats (for review (Young, 1999)).

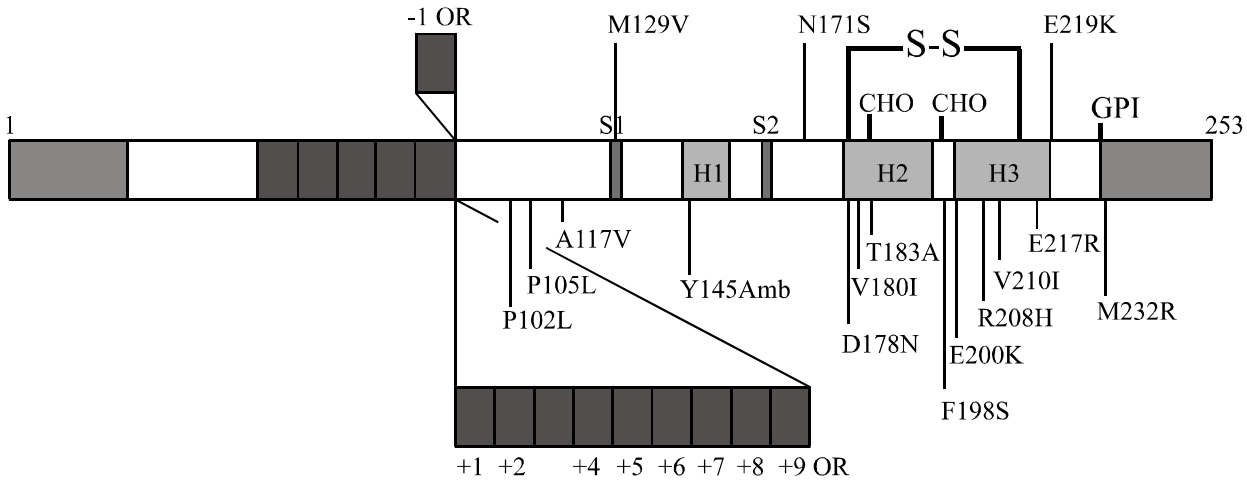

Fig. 2: Structural features and mutations and polymorphisms of the human prion protein. The mutations indicated below represent point mutations. The wild-type amino acid precedes the mutant amino acid. Polymorphisms are indicated above the prion protein. OR: octarepeat region, CHO: glyocosylation sites, Amb: amber codon – termination codon, GPI: glycosyl-phosphatidylinositol anchor, S1, S2: β-sheet 1 and 2, H1, H2, H3: α-helix 1, 2 and 3.

The octarepeat region is a region located within the unstructered N-terminus (aa 51-91) consisting of a fivefold repeat (in human) of eight amino acids which are glycine and proline rich (Kretzschmar *et al*., 1986).

The GSS phenotype is denoted by a slower progression of the disease in which ataxia is the predominant sign together with different amyloid deposits (Ghetti *et al*., 1995). The following mutations are responsible for GSS: P102L, P105L, A117V, F198S and E217R.

Fatal familial insomnia (FFI) is associated with one mutation: D178N. The typical hallmarks of the FFI thalamic degeneration together with atrophy (Medori *et al*., 1992) is only detectable when methionine is encoded at position 129. Valine at position 129 together with D178N results in familial CJD.

2. The prion protein

2.1. Processing and structure of PrP

The ORF of the human *Prn*-p gene is located in one exon on chromosome 20 and translation results in a protein with a size of 253 amino acids (Fig. 3). On the N-terminus of the precursor form of the prion protein a signal peptide is located which traffics PrP through the endoplasmatic reticulum and the Golgi apparatus to the cell surface. PrP is N-glycosylated at two asparagin residues located in the C-terminal part of PrP (aa 181 and 197) (Bolton *et al*., 1985). Attachment of a glycosylphosphatidylinositol (GPI) anchor occurs at position 231 of the human prion protein (Stahl *et al*., 1987). In addition, a single disulfide bridge is formed between the cysteine residues at position 179 and 214 (Safar *et al*., 1990). The mature human prion protein 23-231 is extracellularly attached to the cell membrane via its GPI anchor. PrP is interacting on the cell surface with its cellular receptor the 37-kDa/67-kDa laminin receptor (Gauczynski *et al*., 2001b). PrP becomes internalized via caveolae-like domains (Vey *et al*., 1996) or clathrin-coated pits (Shyng *et al*., 1994). Since the 37-kDa/67-kDa laminin receptor is required for PrP^c internalization, clathrin coated pits are more likely to mediate the internalization process than caveolae like domains (Gauczynski *et al*., 2001b).

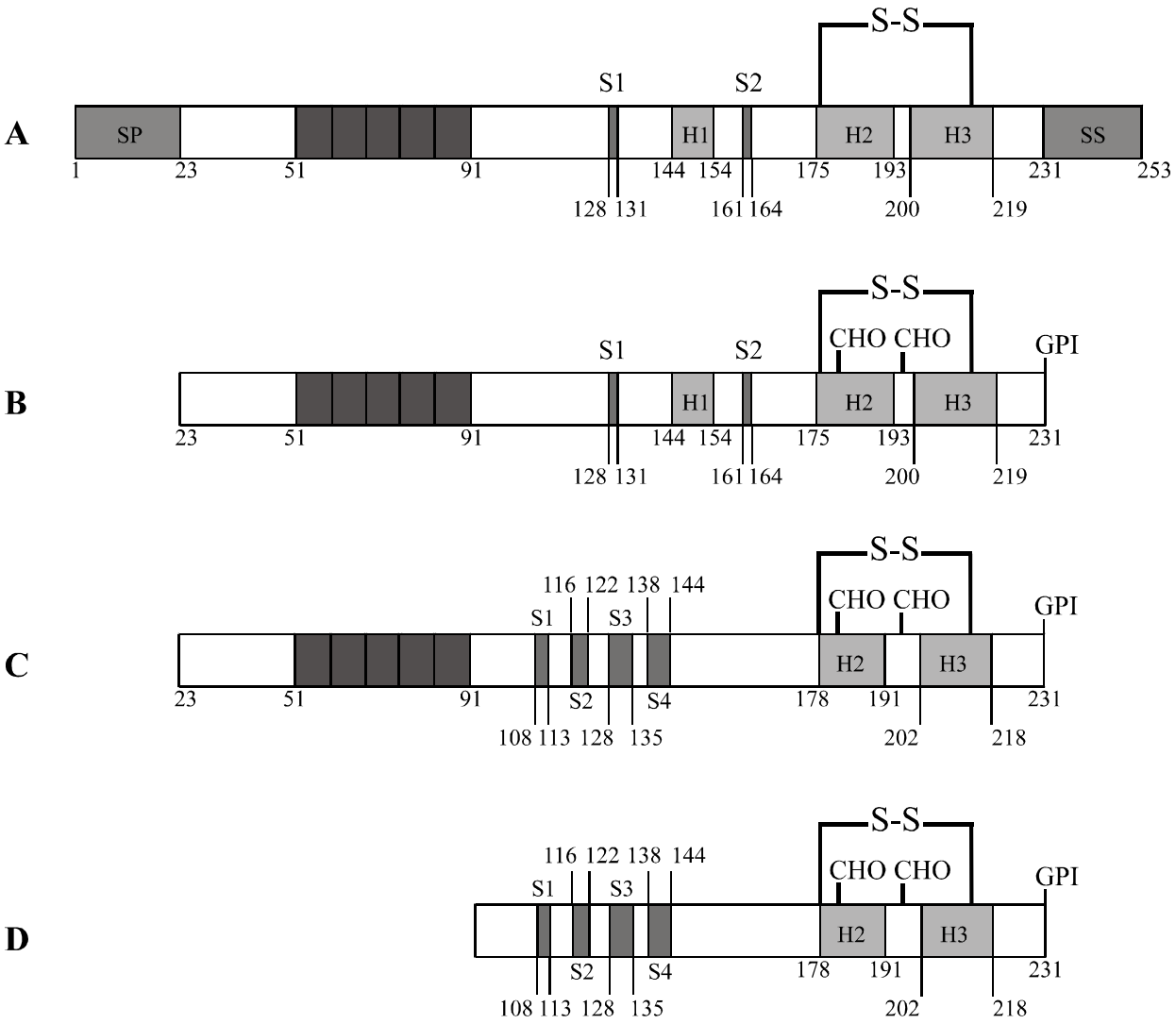

Fig. 3: Maturation of the human prion protein. (**A**) Precursor form of PrP containing the signal peptide at the N-terminus and the signal sequence at the C-terminus. (**B**) Mature form of PrP. The signal domains are cleaved off and the protein is glycosylated and a GPI-anchor is added. (**C**) Converted form of PrP. A model of PrP^{Sc} shows a higher β-sheet content in the N-terminal part (Huang *et al*., 1996). (**D**) Proteinase K resistant part or PrP. After treatment with proteinase K the C-terminal portion of PrP remains undigested. The fragment termed PrP27-30 starts approximately with amino acid 90 (Hope *et al*., 1986). CHO: glyocosylation sites at aa 181 and 197, GPI: glycosylphosphatidylinositol anchor, S1, S2, S3 and S4: β-sheet 1, 2, 3 and 4; H1, H2, H3: α-helix 1, 2 and 3, SP: signal peptide; SS: signal sequence; S-S: disulfide bridge (aa 179 – aa 214). Amino acid numbering of human PrP is indicated.

The human prion protein shows different structural features containing a globular domain extending from residues 125 to 228, where three α-helices and two β-sheets are located as determined by NMR analysis (Fig. 4) (Zahn *et al*., 2000). The N-terminal part of the protein is highly flexible lacking any structural motif.

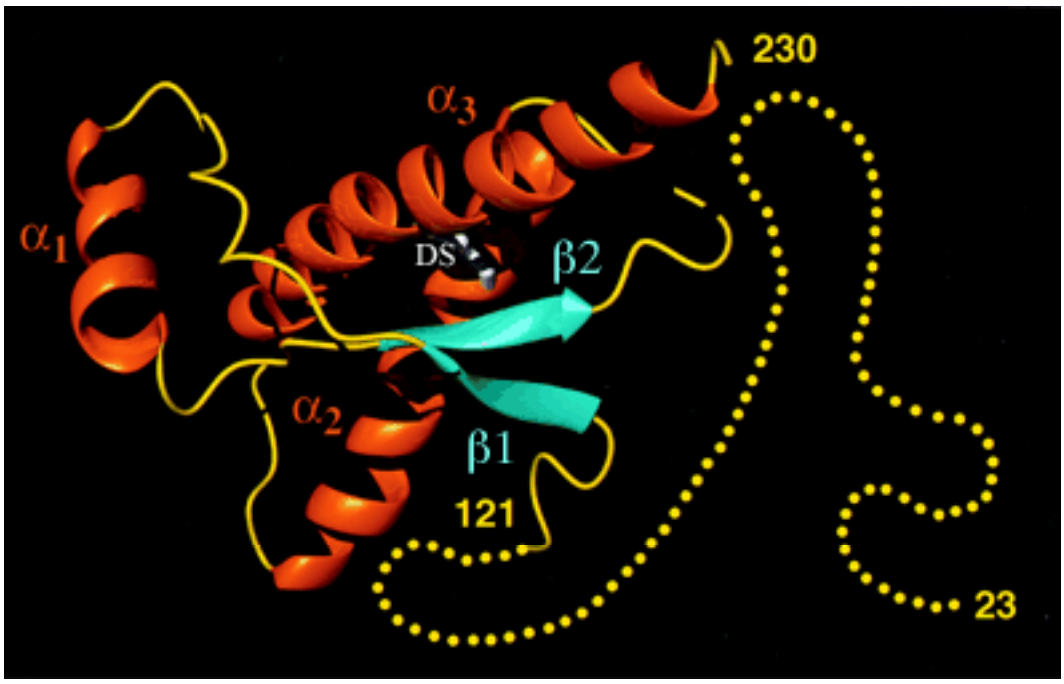

Fig. 4: NMR-structure of the human prion protein residues 23-230 modified from (Zahn *et al*., 2000). The α-helices (curled structures) and β-sheets (arrows) are indicated. In addition, the disulfide bridge is also marked (DS). The flexible unstructured tail of residues 23-121 is represented by yellow dots.

Within the N-terminus a highly conserved region is located consisting of five repeats of an octamer (octarepeats). This region binds copper *in vivo* (Brown *et al*., 1997) and might be important for the function of the prion protein (see chapter I 2.3). Newest results indicate that the crystal structure of the prion protein is dimeric (Knaus *et al*., 2001). The relevance of this finding has to be further investigated due to non-physiological conditions used in this crystalization study.

2.2. Conversion of the cellular prion protein to its pathogenic isoform

The conversion of the cellular form of the prion protein to its pathogenic isoform is the central event of prion diseases. For this reaction and the replication of the pathogen several hypotheses have been proposed. The most accepted one is the „protein-only“ hypothesis (heterodimer hypothesis) proposed by S. B. Prusiner (Prusiner, 1991). The cellular prion protein (PrP^{c}) interacts with the scrapie form (PrP^{Sc}) forming a heterodimer, in which the PrP^{c} is forced to adopt the pathogene structure. The newly generated PrP^{Sc} is able to transform

further PrP^c molecules resulting in a chain reaction which leads to death of the organism. The conversion reaction has been successfully performed *in vitro* (Caughey *et al*., 1995), but the *in vitro* generation of infectious material failed. This leads to the conclusion that an additional factor termed protein X might be necessary for the formation of pathogenic prions (Telling *et al*., 1995). Most promising candidates for protein X represent chemical (Tatzelt *et al.*, 1996b) or molecular (Edenhofer *et al.*, 1996) chaperones, which may favour the structural changes during the conversion reaction (Liautard, 1993). A further model for the conversion of PrP^c into PrP^{Sc} is the nucleation-dependent polymerization model (Lansbury and Caughey, 1995). Here, a so called crystal seed induces the conversion reaction. During the conversion process α-helices of the cellular PrP are thought to change into a β-sheeted structure (Fig. 3) (Huang *et al*., 1996; Pan *et al*., 1993).

The change in the secondary structure has dramatic effects on the properties of the prion protein. The cellular PrP is soluble and completely sensitive towards proteinase K treatment (Prusiner *et al*., 1981). In contrast, PrP^{Sc} has a high tendency to aggregate and PK treatment results in a fragment of the protein termed PrP27-30, lacking about 60 amino acids at the N-terminus compared to full-length PrP^{Sc} (Fig. 3). Like full-length PrP^{Sc} PrP27-30 is also extremely resistant towards agents such as formamide or UV-radiation (Meyer *et al*., 1986). The pathogenic PrP can be inactivated by phenol, NaOH or heat under pressure (138°C, 3 bar >20 min). This high resistance of the scrapie form of the prion protein against degradation leads to aggregation into scrapie-associated fibrils (SAFs) in the cells (Prusiner *et al*., 1983) resulting in cell death.

2.3. Function of PrP

The question about the function of the cellular prion protein is still speculative. The generation of mice ablated of the prion gene did not provide any prominent indications about the function of PrP. The first *Prn*-p deleted mice were generated in 1992 and showed no significant phenotype compared to wild-type mice (Bueler *et al*., 1992). This finding was confirmed by other studies employing $PrP^{0/0}$ mice (Manson *et al*., 1994). Other $PrP^{0/0}$ mice showed some alterations in their circadian rhythms and sleep behaviour (Tobler *et al*., 1996) whereas another mouse line indicated the most intriguing features which suggested a participation of PrP in the long-time survival of Purkinje neurons displayed in the development of ataxia (Moore *et al*., 1999). This phenotype. however, is not due to the deletion of the *Prn*-p gene, but to the hyperexpression of doppel encoded by the *Prn*-d gene located 3‘ of the *Prn*-p locus. Some reports employing *Prn*-p knock-out mice describe a role

of PrP^c in synaptic processes (Collinge *et al*., 1994). The only confirmed function of PrP is its necessity for the infection with prions. Mice homozygous deleted in the *Prn*-p gene were not infectable whereas heterozygous mice were only partially protected against scrapie infection indicated in a prolonged incubation period (Bueler *et al*., 1993).

The role of PrP in the copper metabolism has been postulated: copper ions bind to the octarepeat region located at the flexible N-terminus with micromolar affinity via coordination with four histidins (Viles *et al*., 1999; Brown *et al*., 1997). Copper is an essential metal, which plays a fundamental role in cell biochemistry, involving catalytic activities of several enzymes including the superoxide dismutase (SOD). Cells expressing high levels of PrP showed an increased resistance to oxidative stress compared to PrP knock out cells indicating an increased Cu/Zn SOD activity (Brown and Besinger, 1998). *Prn*-p ablated mice showed a reduced SOD activity in the brain (Wong *et al*., 2000). Whether this observation is due to an increased copper transport of PrP to cuproenzymes such as SOD-1 or in the SOD activity of PrP, which was reported for recombinant and immunoprecipitated PrP from mouse brains (Brown *et al*., 1999), remains speculative. Copper homeostasis and oxidative stress are relevant for the normal function of the CNS including synaptic transmissions. Any disturbances in the copper transport or homeostasis are linked with some neurodegenerative diseases such as the Menkes‘ syndrome, Wilson disease and Alzheimer’s disease (Waggoner *et al*., 1999).

Recently, the involvement of PrP in signal transduction by activation of tyrosine kinase Fyn has been reported (Mouillet-Richard *et al*, 2000) suggesting a possible role of PrP in signal transduction pathways (see chapter I 4.).

3. The prion-like protein Doppel

The prion-like protein Doppel (Dpl) was discovered during sequencing of a genomic DNA cosmid clone isolated from mice (Moore *et al*., 1999). ″Doppel″ is an acronym derived from *do*wnstream *p*rion *p*rot*e*in-*l*ike gene. The mouse doppel gene *Prn*-d locates about 16 kb downstream of the *Prn*-p coding region and is conserved in different strains of mice (Moore *et al*., 1999) and in other mammals including sheep and cattle (Tranulis *et al*., 2001). With respect to gene structure the doppel gene is in contrast to *Prn*-p interrupted by one intron which leads to two mRNAs as alternative splice variants 2,7 and 1,7 kb in size, respectively (Moore *et al*., 1999). The expression pattern of PrP and Dpl is quite different. Whereas PrP is

expressed in a variety of neuronal tissues including brain, Dpl mRNA and the Dpl protein were found in adult testis and heart, but were absent in the CNS of wild-type mice (Silverman *et al*., 2000). In contrast, the expression of Dpl was upregulated in the brain in mice ablated of the prion gene (Moore *et al*., 1999).

Comparison of the sequence of PrP with Dpl reveals a homology of 25% between both proteins. On the first glance, Dpl seems to be an N-terminally truncated version of PrP lacking the octarepeat region which might contribute to the function of PrP (Moore *et al*., 1999). In addition Dpl also lacks the highly conserved hydrophobic region within the core region of PrP, which might represent a putative transmembrane region. Both proteins harbor the N-terminally located signal peptide responsible for translocation of the proteins through the secretory pathway. Both proteins reveal also a signal sequence necessary for the addition of the GPI-anchor. Dpl is an asparagin-linked glycosylated protein analogous to PrP, which exist in non-, mono- and di-glycosylated forms (Silverman *et al*., 2000). As expected from the existance of a signal sequence a GPI-anchor is added to the Dpl protein allowing the fixation to the cell membrane on the cell surface. This was proven by a PIPLC treatment, which cleaves off GPI-anchored proteins from the cell surface (Silverman *et al*., 2000). One major difference between both proteins is the existance of an additional intramolecular disulfide bridge for Dpl identified by mass spectrometrie (Lu *et al*., 2000). The solution of the NMR-structure of Dpl revealed that Doppel and PrP share about the same features (Mo *et al*., 2001). Three α-helices and two short β-strands are present in both proteins, whereas some interstructural kinks and loops are different (Fig. 5).

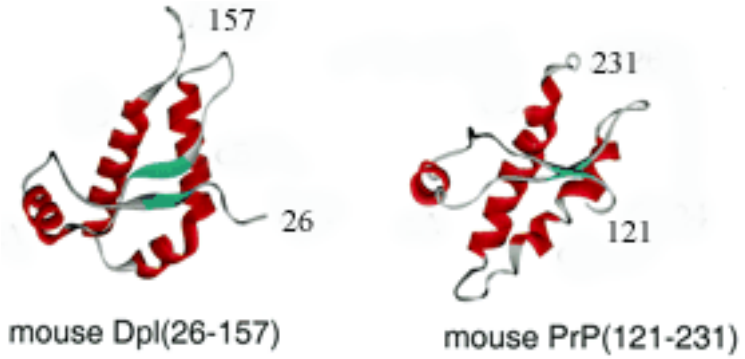

Fig. 5: NMR-structrures of mouse Dpl 26-157 and mouse PrP 121-231 (modified from (Mo *et al*., 2001)). Each structure reveals three α-helices and two β–sheets.

The role of Dpl in prion pathogenesis replication remains unclear to date. Dpl cannot be detected in the CNS, the main location of prion replication. The typical features of prion pathology including spongiosis, gliosis and PrP^{Sc} accumulation were still observed in *Prn*-d deficient neuronal tissues inoculated with scrapie prions (Behrens *et al*., 2001). Therefore, Dpl seems to be likely dispensable for prion disease progression and generation of PrP^{Sc}.
Recently, the overexpression of Dpl in the CNS of transgenic mice on a *Prn*-$p^{0/0}$ background has no effect on the incubation time of a scrapie infection (Moore *et al*., 2001). In addition, we were able to show that Dpl also fails to interact with PrP and the laminin receptor, the receptor for cellular PrP (see chapter VI). Whether Dpl represents the proposed molecule (Shmerling *et al*., 1998) which might be responsible for signal transduction in the absence of PrP necessary for cell survival has to be further investigated.

4. Participation of PrP in signal transduction

Due to its cell membrane localization the cellular prion protein could participate in cell signalling pathways. There are some reports about the involvement of PrP in cellular calcium signalling pathway. PrP contains a 21-amino acid fragment (PrP106-126) which showed a cytotoxic effect on neuronal cultivated cells (Forloni *et al*., 1993). Two possibilities might be responsible for this phenomenon. An interaction of the cytotoxic fragment with an intrinsic ion transport protein or the formation of an ion channel are conceivable models (Kourie and Shorthouse, 2000). The fragment PrP106-126 is able to form ion channels in planar bilayers that were permeable to common physiological ions (Lin *et al*., 1997). This might disturb the ion balance of the cell leading to apoptosis. In fact the prion protein affects the Ca^{2+}/K^{+} homeostasis in cerebellar Purkinje cells (Herms *et al*., 2001). PrP is proposed to have superoxide dismutase activity which is reduced in *Prn*-$p^{0/0}$ neurons (Brown *et al*., 1999). The SOD acitivity is predominantly involved in reduction of intracellular produced oxygen radicals which are known to affect the Ca^{2+} influx (Guerra *et al*., 1996). Therefore, it is unknown whether the cell death of PrP knockout cells is due to a lack of SOD-activity and copper transport or the disturbance of the Ca^{2+} homeostasis. Calcium is an important player in the signalling pathways of cells functioning as a second messenger (Berridge, 1998), which can be activated by PrP. The transient release of intracellular calcium is a result of a stimulation of microglial cultures by fibrillar neurotoxic prion peptides (Combs *et al*., 1999). This effect is also observed with Aβ-peptide in the Alzheimer's disease another

neurodegenerative disease which is as well characterized by amyloidogenic features (Kourie and Henry, 2001). The fibrillar peptides activate tyrosine kinases Lyn and Syk which initiate a signalling cascade involving calcium as a second messenger. In addition, the involvement of tyrosine kinases including Src kinases Fyn and Lck in the mobilization of intracellular Ca^{2+} via phosphilipase-C and IP_3 is reported (Archuleta *et al*., 1993).

Some further evidences exist for the connection of the prion protein with the signal pathways involving tyrosine kinases. PrP, the tyrosine kinases Fyn and Yes and subunits of the G protein were located in the same caveolin-free complexes isolated from neuroblastoma cells (Gorodinsky and Harris, 1995). In addition, the neurotoxic prion peptide fragment PrP 106-126 behaves chemotactic for human monocytes through the use of a G-protein-coupled formyl peptide receptor-like-1 protein (FPRL-1) activating phagocytosis (Le *et al*., 2001a; Le *et al*., 2001b). Src kinases were also involved in the phagocytotic process mediated by the kinase ZAP-70 (Majeed *et al*., 2001; Park and Schreiber, 1995). There is another common feature for the neurotoxic PrP fragment and Fyn kinase in the relationship with the mitogen-activated protein kinase (MAPK). PrP 106-126 activates the MAPK during the induction of nitric-oxide synthase (Fabrizi *et al*., 2001), whereas Fyn plays a role in the signal cascade of the activation of MAPK by thrombin in T cells (Maulon *et al*., 2001). In addition, scrapie infection of neuroblastoma cells provoked up-regulation of the insulin-like growth factor-1 receptor (IGF-1R) (Ostlund *et al*., 2001) probably mediated by Src family tyrosine kinases which are known to participate in the signalling of IGF-1R (Boney *et al*., 2001).

Recently, a more direct relation of the prion protein with the tyrosine kinase Fyn was described (Mouillet-Richard *et al*, 2000). It was demonstrated that the activity of Fyn was strongly increased in murine neuronal cells by antibody mediated cross-linking of PrP^c. Since Fyn is located on the innerside of the cell membrane and PrP stays GPI-anchored on the cell surface, PrP is a potent candidate for signal transduction to modulate the signals from other cells or the extracellular space. Therefore, an additional factor might be necessary to mediate the signal from PrP to Fyn. A potential candidate is the 37-kDa/67-kDa laminin receptor (LRP/LR) acting as the receptor for cellular PrP (Gauczynski *et al*., 2001b). The receptor is a transmembrane receptor class 2 with its C-terminus orientated to the extracellular space (Gauczynski *et al*., 2001b). Some other factors might also be involved in this process, e.g. heparan sulfate proteoglycans (HSPGs), which are binding partners for PrP and LRP/LR (Hundt *et al*., 2001), caveolin-1 or clathrin (Mouillet-Richard *et al*, 2000). Wether phosphorylation of PrP, which is susceptible for enzymic phosphorylation by tyrosine kinases (Negro *et al*., 2000) in combination with structural changes of PrP, or a more indirect way of

signal transduction mediated by PrP is speculative to date. However, the important role of PrP in signal transduction has been confirmed by the detection of several other proteins such as Grb2 and Synapsin Ib interacting with PrP involved in signalling pathways (Spielhaupter and Schatzl, 2001).

5. Yeast two-hybrid system

Protein-protein interactions are crucial steps in many biological processes, such as signal transduction pathways or receptor/ligand interactions. A useful tool for the identification and analysis of protein-protein interactions represents the yeast two-hybrid system, which was first developed by Fields and Song in 1989 (Fields and Song, 1989). The basic concept of the yeast two-hybrid system emerged from the analysis of transcription factors which bind to DNA sequences upstream the target genes followed by activation of the transcription machinery (Vidal and Legrain, 1999). DNA binding and activation features are located in physically separable domains of the protein (Keegan *et al*., 1986) which can be fused to proteins of interest. Only in case the proteins interact with each other the transcription factor is reconstituted and a reporter system is switched on (Fig. 6). In addition to the first reporter gene which was the bacterial *lacZ* gene further selection markers such as LEU have been established leading to growth of the yeast cells indicating the protein-protein interaction (Gyuris *et al*., 1993).

5.1. Modifications of the yeast two-hybrid system

In the last few years, several new modifications of the yeast two-hybrid system have been established recommanding the system for several different approaches. In order to detect factors mediating the interaction of two proteins a three-hybrid system was generated involving a third component such as a nucleic acid or a protein (Tirode *et al*., 1997). The reverse two-hybrid has been generated to select for mutations, drugs or competing protein disrupting a protein-protein interaction (Vidal *et al*., 1996). The readout of the reverse two-hybrid system is inverse to the regular two-hybrid system: yeast cells are only viable in case of no protein/protein interaction. A similar selection is performed in case of the split hybrid system, where the *tet* repressor system is used for selection (Shih *et al*., 1996).

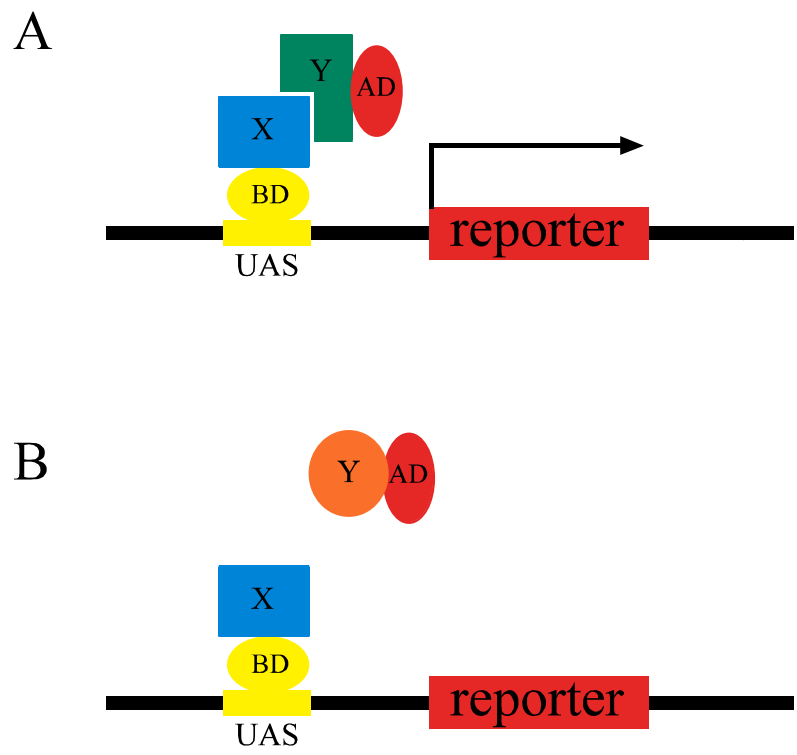

Fig. 6: Principle of the two-hybrid system. Protein X is fused to a DNA-binding domain (BD), which is capable to bind to the upstream activating sequence (UAS), e.g. LexA or GAL4, whereas protein Y is fused to an activation domain (AD), e.g. the acidic activation domain B42. **A** If protein X interact with protein Y, the transcription factor is reconstituted and the reproter gene (*lacZ* / HIS3 / LEU2) is switched on. **B** No interaction between protein X and Y leads to an inactive reporter gene.

The yeast two-hybrid method has been transfered to the *E.coli* system, which allows the usage of the advantages of a prokaryotic system (for review (Hu *et al*., 2000)). *E.coli* cells grow faster than yeast cells, eukaryotic regulatory proteins interfering the endogeneous yeast metabolism can be investigated in *E.coli* and it is unlikely that *E.coli* proteins hamper this protein-protein interaction.

In addition, the yeast two-hybrid system has been adopted to mammalian cells detecting protein-protein interaction in an evolutionary highly developped system (Serebriiskii *et al*., 2001).

Whereas the regular two-hybrid occurs in the nucleus of the cells in which transcription takes place new methods were established basing on interactions in the cytoplasm involving

ubiquitin restoration (Johnsson and Varshavsky, 1994) or membrane-association employing the Ras signalling pathway (Aronheim *et al*., 1997).

There is a broad application of the two-hybrid system for the investigation of the protein network of a cell. Potential binding partners can be identified in a two-hybrid screen using a cDNA library encoding many proteins. The regions involved in the interaction can be determined by mapping analyses employing deletion or insertion mutants. Considering the knowledge of the genome of different organisms cloning of open reading frames of part of the genome and investigation in a two-hybrid analysis is possible (Hudson *et al*., 1997). Two-hybrid technology plays also an important role in the proteomic studies of genomes, in which besides mass spectrometry the two-hybrid technology represents a powerful tool for the detection of protein-protein interactions (Pandey and Mann, 2000).

5.2. Yeast two-hybrid analyses with the prion protein

A series of proteins interacting with the prion protein have been identified in two-hybrid analyses (for summary see chapter II). In 1995, Bcl-2 was the first PrP interacting protein detected in a yeast two-hybrid screen (Kurschner and Morgan, 1995), including the identification of ihnteraction domains (Kurschner and Morgan, 1996). The molecular chaperone Hsp60 was identified as an interactor for human PrP favouring the hypothesis that these molecules participate in the progression of TSEs (Edenhofer *et al*., 1996). In addition, the 37-kDa/67-kDa laminin receptor was discovered as an interactor for PrP (Rieger *et al*., 1997). This receptor has been proven to act as the receptor for the cellular prion protein (Gauczynski *et al*., 2001b) and PrP/LRP-LR interaction domains have been also identified (Hundt *et al*., 2001). Very recently, the participation of the prion protein in signaling pathways has been proposed from yeast two-hybrid analyses demonstrating the interaction of PrP with Grb2 and Synapsin Ib (Spielhaupter and Schatzl, 2001).

Interacting proteins of the prion-like protein Ure2p, a non-Mendelian genetic element of the yeast *Saccharomyces cerevisiae* (for review (Tuite, 2000)), have also been identified and characterized by a yeast two-hybrid analyses (Komar *et al*., 1999; Fernandez-Bellot *et al*., 1999).

Chapter II

Interaction of prion proteins with cell surface receptors, molecular chaperones, and other molecules.

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I. Introduction

The prion protein PrP represents a central player in transmissible spongiform encephalopathies (TSEs), also known as prion diseases (for review see Lasmézas and Weiss, 2000). The physiological role of the cellular isoform of PrP termed PrP^c is speculative so far (for review see (Weissmann, 1996)) and might involve control of circadian activity rhythms and sleep (Tobler *et al*., 1996), maintenance of cerebellar Purkinje cell (Sakaguchi *et al*., 1996), and normal synaptic functions (Collinge *et al*., 1994; Fournier *et al*., 1995; Kitamoto *et al*., 1992). Because several reports do not describe any phenotype for PrP (Bueler *et al*., 1992; Lledo *et al*., 1996; Manson *et al*., 1994) the only proved role of PrP^c is its necessity for the development of TSEs (Bueler *et al*., 1993) such as bovine spongiform encephalopathy (BSE) in cattle, new variant Creutzfeldt-Jakob (nvCJD) in humans or scrapie in sheep. A recent report describes a superoxide dismutase (SOD) activity for PrP^c (Brown *et al*., 1999) suggesting that PrP might play a role in the cellular resistance to oxidative stress.

In the last 20 years of the past twentieth century, researchers worldwide were eagerly searching for molecules able to interact specifically with the prion protein in the hope of identifying interactors (1) that play an important role in the life cycle of prions or (2) that could be developed into powerful TSE therapeutics.

This chapter summarizes PrP interacting molecules that might be relevant for PrP pathogenesis or TSE therapy. In the first section we describe putative prion protein receptors including the role of heparan sulfate proteoglycans (HSPGs). A cellular model will be presented that describes the possible role of prion receptors and prion proteins, including the recently identified PrP-like protein termed doppel (Moore *et al*., 1999). The model emphasizes the possible role of PrP and its receptor regarding PrP internalization as well as signal transduction and physiological function, in particular, the 37 kDa laminin receptor precursor (LRP), an up to now unidentified 66 kDa cell surface protein, and cadherins, which are then discussed as prion receptors that might trigger the entry of PrP into scrapie infectable cells. Next, we summarize the role of molecular chaperones, including chemical chaperones that may catalyze or hamper the conversion process of PrP^c to PrP^{Sc}. In this context, we emphasize a possible function for protein X, an as yet unknown protein predicted by S.B. Prusiner to be necessary for the PrP conversion process. The occurrence of PrP dimers under native and denaturing conditions observed in different cell systems and *in vitro* represents another aspect of PrP interactions, in this case an interaction of PrP with itself. The possible role of such PrP dimers in the complex scenario of PrP oligomerization and multimerization

processes is discussed. In the section V we report on a series of PrP interacting molecules identified using different biochemical approaches such as ligand blotting and yeast two-hybrid techniques. Among these are the PrP ligand proteins (Pli) encompassing Pli 3-8, Pli 45 and 110 as well as Bcl-2, which belongs to a family of proapoptotic and antiapoptotic molecules. The role of Bcl-2 in the light of neurodegeneration and apoptosis is discussed. The interaction between laminin and PrP-mediating neuritogenesis is reported. The last section describes molecules, mainly of nonproteinaceous origin, which act as therapeutics for the treatment of TSEs. These include polyanions such as heteropolyanion 23, dextran sulfate 500, pentosan polysulfate (SP54), and heparin. Other groups of anti-TSE therapeutics include Congo red, polyene antibiotics such as AmB and MS-8209, IDX, porphorins, phtalocyanes and the protein clusterin. The possible modes of action of these molecules such as interfering with the PrP^{c}/PrP^{Sc} conversion process followed by PrP accumulation, interfering with the cellular uptake of PrP^{c}/PrP^{Sc}, overstabilization of PrP^{Sc}, or competing with cellular glycosamino-glycans for the binding to PrP^{c} are discussed. The last group of PrP interacting molecules represent nucleic acids including RNA aptamers, the latter as a possible tool for the diagnosis of TSEs.

II. Cell Surface Receptors

A. The Role of a Cellular Prion Protein Receptor

To understand the pathogenesis of diseases such as TSEs, it is necessary to clarify how the biological system works under physiological conditions. The main principle of the "protein-only“ hypothesis is that the cell-membrane glycoprotein PrP^{c} is converted into its pathogenic isoform PrP^{Sc}, a process that involves conformational changes of the protein (Prusiner *et al*., 1998). During this transformation PrP acquires additional regions of ß-sheets in the polypeptide chain, resulting in a partially resistance to proteases. The cellular pathway of PrP^{c} is of major interest because here the conversion of PrP^{c} to PrP^{Sc} might take place. PrP^{c} is synthesized in the rough endoplasmatic reticulum (rER). It is passaged via the Golgi and secretory granules to the cell surface where it is anchored to the plasma membrane by its glycosylphosphatidylinositol (GPI) moiety (Rogers *et al*., 1991). According to an endocytic recycling pathway, the surface-PrP^{c} is internalized by clathrin-coated pits (Shyng *et al*., 1994) or caveolae-like domains (CLDs) (Vey *et al*., 1996). The endocytosis of PrP^{c} could be mediated by a transmembrane protein, which might connect the GPI-anchored PrP to clathrin.

Harris postulated the existence of an endocytic PrP-receptor that carries a coated-pit localization signal in its cytoplasmic domain and whose extracellular domain binds the N-terminal part of PrP^c (Harris, 1999; Harris *et al*., 1996). He observed that deletions within the N-terminal region of PrP^c result in a decrease of internalization of the protein and consequently in a reduction of the PrP^c concentration in coated pits (Harris, 1999; Shyng *et al*., 1995). In addition, Harris observed that chicken PrP binds to the surface of mammalian cells via heparan sulfates on the cell surface (Shyng *et al*., 1995). Several researchers described an interaction between heparan sulfates and PrP (Brimacombe *et al*., 1999; Caughey *et al*., 1994; Chen *et al*., 1995; Gabizon *et al*., 1993). Heparan sulfates have been shown to be a component of amyloid plaques in prion diseases (Gabizon *et al*., 1993). Recently, it has been demonstrated that the addition of heparin competes with the binding of copper to PrP which occurs in the octarepeat region (Brown *et al*., 1997; Brimacombe *et al*., 1999), suggesting that this region of PrP binds to heparin. The recently observed superoxide dismutase (SOD) activity of PrP^c is dependent on the presence of the octarepeat region (Brown *et al*., 1999) confirming the important role of this domain for PrP. HSPGs make up proteoglycan moieties consisting of proteins carrying glycosaminoglycan (GAGs) chains made of anionic polysaccharide chains. Heparan sulfate, the main GAG-constituent of HSPGs, like heparin, consists of disaccharide repeating units of O-/N-sulforyl and N-acetylglucosamine (or N-acetylgalactosamine) and O-sulforyliduronic acid except that it harbors fewer N- and O-sulfate groups and more N-acetyl groups. The proteoglycans HSPGs are thought to play an important role on the cell surface within the life cycle of prions.

The process by which exogenous PrP^{Sc} enters the cell is unclear so far. The uptake of the infectious agent could also be mediated by a receptor protein or might occur receptor independent. The conversion of PrP^c to PrP^{Sc} may take place after internalization in cellular compartments such as endosomes, lysosomes, or endolysosomes. This conversion process is thought to be influenced by an unknown protein termed protein X (Telling *et al*., 1995), which could represent a molecular chaperone such as Hsp60 (Edenhofer *et al*., 1996). In addition, it has been suggested that several proteins possessing a GPI-anchor are excluded from coated pits and internalized by caveolae (Anderson, 1993). Furthermore, it has been reported that PrP^c and PrP^{Sc} are present in CLDs isolated from scrapie-infected neuroblastoma cells and brains of scrapie-infected hamsters, and it is speculated that the conversion of PrP^c into PrP^{Sc} could also take place in these compartments (Vey *et al*., 1996). To understand the mechanism of this conversion event as well as the physiological function of the cellular prion protein, it is important to investigate the involvement of a possible receptor protein as well as

of proteins showing biological properties similar to PrP, such as the recently discovered PrP-like protein designated doppel (Dpl) (Moore *et al*., 1999).

The discovery of doppel does not only represent the first PrP-related protein (Moore *et al*., 1999), it also could explain some curious, surprising observations within several lines of $Prnp^{0/0}$ mice, which differ only in the strategy used to generate PrP^{c}-deficiency. Creating an internal insertion or deletion within the PrP exon 3, two lines of mice were generated showing normal development without any pathological phenotype (Bueler *et al*., 1992; Lledo *et al*., 1996; Manson *et al*., 1994). However, in two other cell lines the entire coding sequence of PrP as well as a ~1 kb region 5' to exon 3 including the exon 3 splice acceptor site were deleted (Sakaguchi *et al*., 1996). These $Prnp^{0/0}$ mice showed progressive symptoms of ataxia and Purkinje cell degeneration in the cerebellum. It is suggested that Dpl is involved in a physiological process in a manner leading to this pathological phenotype. Doppel is the first PrP-like protein to be described in mammals (Moore *et al*., 1999). It consists of 179 amino acid residues showing ~25 % identity with all known prion proteins. The Dpl locus, *Prnd*, is located 16 kb downstream of the PrP gene, *Prnp*, generating two major transcripts of 1.7 and 2.7 kb. Like PrP, Dpl mRNA is expressed during the embryogenesis but, in contrast to PrP, it is poorly expressed in the adult central nervous system (CNS) and at high levels in the testis of mice. However, Dpl is upregulated in the CNS of the two $Prnp^{0/0}$ lines that develop late-onset ataxia and Purkinje cell death but not in the normally developed $Prnp^{0/0}$ lines (Moore *et al*., 1999). Therefore, it was assumed that Dpl may provoke neurodegeneration in PrP-deficient mice, an observation that might explain why some lines of $Prnp^{0/0}$ mice develop cerebellar dysfunction and Purkinje cell death, whereas others do not. Moore *et al*. suggested that Dpl and PrP may share some biological functions owing to the similarities between these two proteins (Moore *et al*., 1999). Would it be possible that PrP and Dpl bind to each other or would it be also possible that they compete for binding to a common receptor? Dpl synthesis is thought to occur in the secretory pathway to yield a globular, N-glycosylated, membrane-associated protein comparable to PrP^{c}, but in contrast to it containing no octarepeat region in its N-terminal domain (Moore *et al*., 1999).

In addition, expression of moderate levels of N-terminal truncated PrP with deletions of amino acid residues 32-121 or 32-134 caused ataxia and specific degeneration of the granular layer of the cerebellum in $PrP^{0/0}$ mice, whereas mice expressing shorter truncations of PrP, up to residue 106, show no pathological changes (Shmerling *et al*., 1998). This granule cell dysfunction was completely abrogated by introducing a single copy of a wild-type murine PrP gene into mice. It is speculated that the truncated PrP may compete with some other molecule

with a function similar to that of PrP for a common ligand or receptor. It was assumed that in wild-type mice PrP interacts with a presumed receptor promoting signal transduction (Fig. 1A), and the same signal is elicited by interaction of the receptor with π, a conjectural protein that has the functional properties of PrP, but is not closely related to it on DNA level (Fig. 1B) (Shmerling *et al*., 1998). This would explain why the absence of PrP^c has no obvious phenotypic consequences. It is postulated that truncated PrP can interact with the receptor without giving rise to a signal (Fig. 1C). The affinity of the receptor for truncated PrP would have to be stronger compared to π, but would be less compared to intact PrP. Only N-terminal truncated PrP where the deletion extends to or beyond residue 121 shows cerebellar dysfunction leading to the conclusion that the globular domain of cellular PrP binds to a receptor, whereas the flexible tail of the N-terminus spanning residues 23 to 120 is

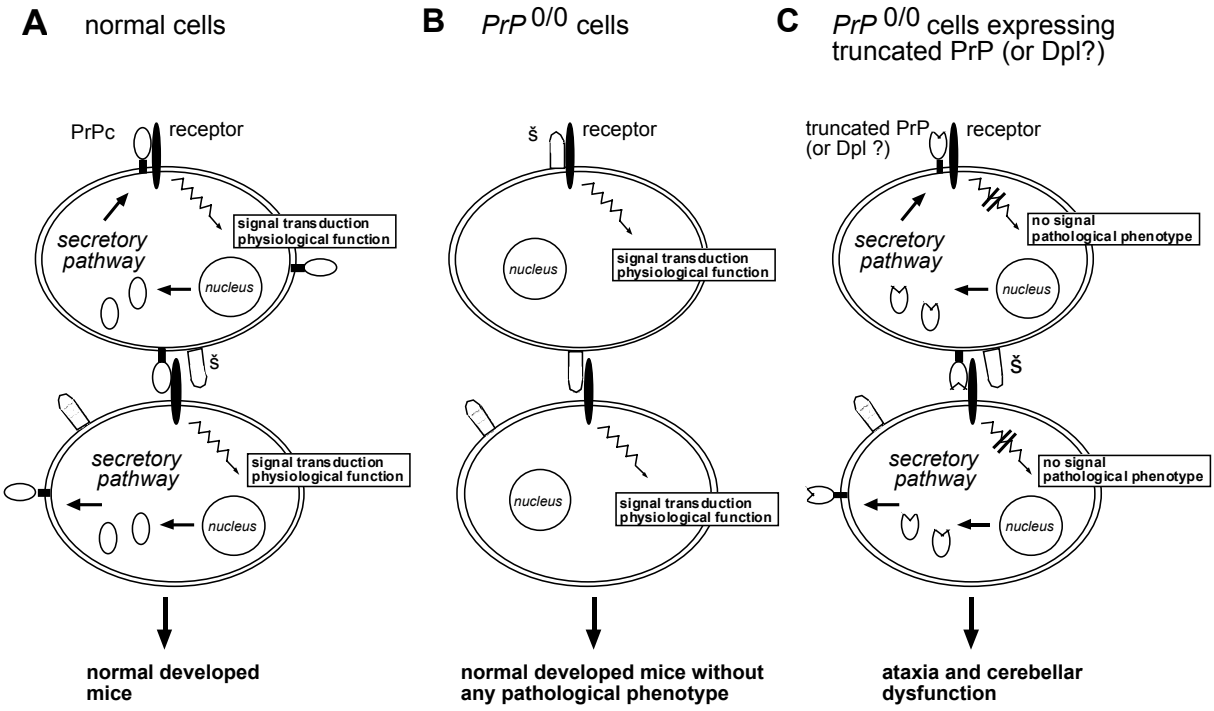

Fig. 1: Model of PrP^c- and receptor-mediated signal transduction. In the normal cell, PrP^c and receptor molecules from the same cell or from different cells can interact and promote signal transduction (A). The same signal might be elicited by the binding of a conjectural protein designated π, which possesses the functional properties of PrP^c explaining why some lines of $PrP^{0/0}$ mice develop normally (B). In the absence of PrP^c, N-terminal truncated PrP can also interact with the receptor competing with the binding of π, however, without giving rise to a signal and leading to ataxia and degeneration of the granular layer of the cerebellum. A similar event is thought to take place in PrP-deficient mice, which are showing a pathological phenotyp. In these mice a PrP-like protein called doppel (Dpl) is upregulated in the CNS. It is speculated that this protein may bind with higher affinity to the receptor than π does, resulting in ataxia and degeneration of Purkinje cells (C).

responsible for activation (Shmerling *et al*., 1998). One possible interpretation for the pathological phenotype caused by the expression of N-terminal truncated PrP is that such PrP-mutants assumes a Dpl-like conformation that is neurotoxic and results in the killing of the granular layer in the cerebellum (Moore *et al*., 1999). The association of Dpl overexpression with degeneration of Purkinje cells which were rescued by overexpression of wild-type PrP, suggest that Dpl and PrP interact perhaps directly or indirectly by competing as ligands for a common receptor. Therefore, both proteins may play a role in cell contact processes (Fig. 1).

Recently, a signal transduction activity of the prion protein by achieving tyrosine kinase Fyn was described (Mouillet-Richard *et al*., 2000). Since PrP^c locates GPI-anchored at the cell surface, whereas Fyn-kinase is associated with the inner plasma membrane of the cell, a transmembrane receptor might mediate the PrP^c dependent activation of the Fyn-kinase.
In this section we describe the different candidates, identified so far, that may act as prion protein receptors. Distinct strategies and methods were used to identify the putative receptor molecule. Further investigations are necessary to clarify the identity of a physiological PrP^c-receptor and to reveal its role in the normal cellular process of PrP^c as well as in the pathogenesis of prion-diseases. Identification and characterization of this receptor are also important in designing drugs that could be used to prevent the initial uptake of the infectious agent into cells.

B. A 66 kDa Membrane Protein as a Potential Prion Receptor

Employing complementary hydropathy a 66 kDa membrane protein that could act as a cellular prion protein receptor, was recently identified (Table I) (Martins *et al*., 1997). By means of this strategy, a hypothetical peptide mimicking the receptor binding site should bind to the neurotoxic domain of prion proteins. Here a peptide encoded by the DNA strand complementary to that of the human PrP gene, spanning amino acid residues 114 to 129, was chemically synthesized and used to immunize mice in order to generate antibodies directed against this complementary prion peptide. The available mouse antisera were used to investigate the localization of the putative receptor by immunofluorescence and confocal microscopy approaches, resulting in the detection of an antigen at the cell membrane of primary mouse neurons. In Western blot analysis of membrane extracts from mouse brain, the antiserum recognized a specific protein of 66 kDa. *In vitro* and *in vivo* binding assays were performed demonstrating that PrP^c and the 66 kDa membrane protein could bind to each other (Martins *et al*., 1997). Flow cytometry studies revealed that purified membrane extracts, prepared from mouse brain, inhibited *in vivo* recognition of cellular PrP in cultured

neuroblastoma cells (N2a) by anti-PrP antiserum. This process could be reversed by pretreatment of such membrane extracts with antiserum raised against the complementary prion peptide and the putative receptor protein. Furthermore, both the complementary prion peptide and the antiserum against it were able to block the neurotoxic effects mediated by the human prion peptide 106-126 towards cultured neuronal cells. Martins *et al*. suggested that a specific receptor for prion proteins could be responsible for their internalization and for the cellular responses mediated by PrP^{c}. They speculated that, as PrP^{c} tends to accumulate in postsynaptic vesicles (Askanas *et al*., 1993), both PrP^{c} and its receptor are involved in interneuronal cell adhesion causing neuronal networking (Martins *et al*., 1997).

According to Martins *et al*. in the normal cell, PrP^{c} and receptors from the same cell or from different cells can interact and mediate signal transduction, triggering their physiological function. They postulated that the infectious agent should interact with the same receptor following internalization, facilitating the conversion of PrP^{c} into PrP^{Sc} and leading to PrP^{Sc} accumulation and finally cell death (Martins, 1999). Further investigations leading to the identification of the 66 kDa protein are necessary to clarify the role of this putative receptor in the normal process of PrP^{c}, as well as in the pathogenesis of TSEs.

Table I. PrP binding proteins, identity and characteristics

PrP binding Protein	cDNA identified	Known homology	Surface protein	Method of identification	Reference
Pli45[c]	Yes	GFAP	No	ligand blot	Oesch *et al*, 1990
Pli110[c]	Yes	PSF	No	ligand blot	Oesch *et al*, 1990
Pli3[c]	Yes	human ESTs	No	PrP-AP screening	Yehiely *et al,* 1997
Pli4[c]	Yes	None	No	PrP-AP screening	Yehiely *et al,* 1997
Pli5[c]	Yes	guinea pig organ of corti, rat and human ESTs	No	PrP-AP screening	Yehiely *et al,* 1997
Pli6[c]	Yes	Mouse *Aplp1* (amyloid precurser like protein)	Yes	PrP-AP screening	Yehiely *et al,* 1997
Pli7[c]	Yes	Mouse *Nrf2* (p45 NF-E2 related factor)	No	PrP-AP screening	Yehiely *et al,* 1997
Pli8[c]	Yes	None	No	PrP-AP screening	Yehiely *et al,* 1997
37-kDa laminin receptor precursor[a]	Yes	37 kDa laminin-receptor precursor	Yes	yeast-two-hybrid screening	Rieger *et al*, 1997
66-kDa protein[a]	No	None	Yes	complementary hydropathy	Martins *et al*, 1997
Cadherins[a]	Yes	Cadherins	Yes	PrP-AP screening	Cashman and Dodelet, 1997
Bcl2[c]	Yes	Bcl-2	No	yeast-two-hybrid screening	Kurschner and Morgan, 1995
Chaperons[b]	Yes	several molecular chaperons	No	various methods	DebBurman *et al*, 1997 Edenhofer *et al*, 1996 Tatzelt *et al*, 1996

[a] See Section II.
[b] See Section III.
[c] See Section V.

C. The 37 kDa Laminin Receptor Precursor (37 kDa LRP)

In a yeast two-hybrid screen, we identified a specific molecule as an interaction partner for the prion protein: the 37 kDa laminin receptor precursor (37 kDa LRP) (Table I) (Rieger *et al*., 1997). We speculated that this protein could act as a potential receptor for the cellular PrP. This interaction was confirmed by coinfection and cotransfection studies in insect and mammalian cells, respectively (Rieger *et al*., 1997). Furthermore, investigations of the LRP level in several organ and tissues of scrapie-infected mice and hamsters demonstrated that LRP occurs in higher amounts only in those organs that exhibit infectivity and PrP^{Sc} accumulation such as brain, spleen and pancreas compared with uninfected control animals (Rieger *et al*., 1997). This was confirmed by cell culture experiments demonstrating an increased amount of LRP in scrapie-infected mouse neuroblastoma (N2a) cells compared with uninfected cells. Mapping of the 37 kDa LRP with different peptide fragments identified a transmembrane domain containing amino acids 86-101 (Castronovo *et al*., 1991b) and a laminin-binding domain comprising amino acids 161-180 (Castronovo *et al*., 1991b), which is thought to be directed towards the extracellular space (Fig.2). Mapping of the LRP/PrP interaction site performed in the yeast two-hybrid system demonstrated that the laminin-binding domain can also function as a PrP binding site (Rieger *et al*., 1997) (Fig. 2).

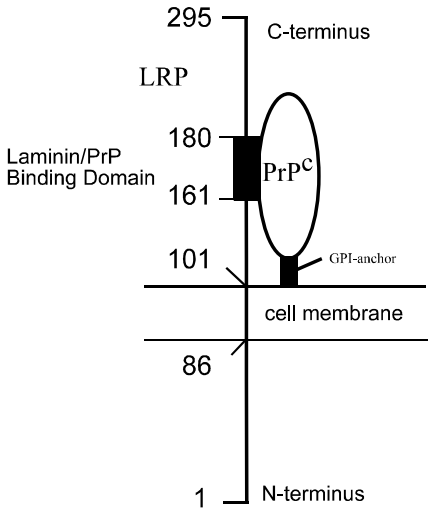

Fig. 2: Schematic view of the prion protein (PrP) and the 37 kDa laminin receptor precursor (LRP) on the surface of a scrapie-infectable cell. PrP is anchored by GPI (Blochberger *et al*., 1997) and is thought to colocalize with LRP. The putative transmembrane region of LRP stretches from aa 86 to aa101 (Castronovo *et al*., 1991b). The laminin binding domains from aa 161 to 180 (Castronovo *et al*., 1991b) encompassing the palindromic sequence LMWWML, which appeared during evolution from the non-laminin-binding ribosomal protein p40 (Ardini *et al*., 1998), to the laminin-binding LRP on the cell surface is identical to the PrP binding domain (Rieger *et al*., 1997).

LRP is thought to be the precursor of the 67 kDa laminin receptor (67 kDa LR) because attempts to isolate the gene for the 67 kDa LR resulted in the identification of a cDNA fragment which encoded a 37 kDa polypeptide (Rao *et al*., 1989; Yow *et al*., 1988). This was confirmed by pulse-chase experiments carried out with antibodies directed against the 37 kDa protein (Castronovo *et al*., 1991; Rao *et al*., 1989). The 67 kDa laminin receptor was first isolated from tumor cells (Lesot *et al*., 1983; Malinoff and Wicha, 1983; Rao *et al*., 1983) owing to its high binding capacity to laminin, a glycoprotein of the extracellular matrix that mediates cell attachment, movement, differentiation and growth (Beck *et al*., 1990). Engelbreth-Holm-Swarm (EHS) laminin (Beck *et al*., 1990), which has been proved to bind to the 37 kDa LRP (Rieger *et al*., 1997) (Table II), consists of three polypeptide chains: A or α (440 kDa), B1 or β, and B2 or γ (each 220 kDa), linked via disulfide bonds, resulting in the typical cross-structure (Beck *et al*., 1990). Several other classes of laminin binding proteins have been described including integrins (Albelda and Buck, 1990) and β-galactoside binding lectins such as galectin-3 (Bao and Hughes, 1995; Ochieng *et al*., 1993; Yang *et al*., 1996) equivalent to CBP-35 (Laing *et al*., 1989). Immunoblotting assays performed with a polyclonal serum directed against galectin-3 revealed that the 67 kDa LR carries galectin-3 epitopes, whereas the 37 kDa LRP does not (Buto *et al*., 1998).

The 37 kDa LRP/67 kDa LR is a multifunctional protein (Table II) and its amino acid sequence is well conserved throughout evolution, showing a high degree of homology among mammalian species (Rao *et al*., 1989). The evolutionary analysis of the sequence identified as the laminin-binding site [which we proved to correspond to the PrP binding domain (Rieger *et al*., 1997)] suggested that the aquisition of the laminin binding capability is linked to the palindromic sequence LMWWML, which appeared during evolution concomitantly with laminin binding (Ardini *et al*., 1998). This protein evolved from the ribosomal protein p40, which participated in protein synthesis on 40 S ribosomes without any laminin-binding activity (Auth and Brawerman, 1992) to a cell surface receptor binding laminin (Rieger *et al*., 1997), elastin (Hinek *et al*., 1988; Salas *et al*., 1992) and carbohydrates (for review see (Ardini *et al*., 1998; Mecham, 1991; Rieger *et al*., 1999)). In addition, interaction of the epitope-tagged laminin binding protein LBP/p40 with nuclear structures was observed in cultured cells (Sato *et al*., 1996). *In vitro* analysis revealed that LBP/p40 binds tightly to chromatin DNA through association with histones H2A, H2B and H4 suggesting that this protein may play an essential role in the maintenance of nuclear structures (Kinoshita *et al*., 1998).

The laminin receptor family is highly conserved in a wide spectrum of eucaryotic cells (Keppel and Schaller, 1991; Wewer *et al*., 1986), including yeast (Demianova *et al*., 1996), and is encoded by archaean genomes (Ouzonis *et al*., 1995). 37 kDa LRP acts as a receptor for the Venezuelan equine encephalitis virus on mosquito cells (Ludwig *et al*., 1996), whereas the 67 kDa LR functions as a receptor for the Sindbis virus on mammalian cells (Wang *et al*., 1992) (Table II). The mechanism of how the 37 kDa precursor protein forms the mature 67 kDa isoform is still unclear. Homodimerization of the 37 kDa LRP (Landowski *et al*., 1995) or the involvement of an additional component (Castronovo *et al*., 1991a) has been discussed. Recent studies suggested that the 67 kDa LR is a heterodimer stabilized by fatty acid-mediated interactions (Buto *et al*., 1998). Very recently, it has been proved that the 67 kDa LR (also termed laminin binding protein, p67 LBP) is expressed on a subset of activated human T lymphocytes and, together with the integrin, very late activation antigen-6, mediates strong cellular adherence to laminin (Canfield and Khakoo, 1999). In summary, the 37 kDa LRP/67 kDa LR polymorphism remains a mystery. Both forms may act as a receptor for prions on the surface of scrapie infectable cells. Mammalian genomes contain multiple copies of the LRP gene, in particular 6 copies in the mouse and 26 copies in the human genome (Fernandez *et al*., 1991; Jackers *et al*., 1996a) a fact that has hampered the identification of the active gene for a long time. To date, only the

gene for the chicken and the human gene encoding LRP have been isolated (Clausse *et al*., 1996; Jackers *et al*., 1996b). The gene encoding 37 kDa LRP belongs to a multicopy gene family and contains seven exons and six introns (Jackers *et al*., 1996b).

The 37 kDa LRP/p40 gene has been identified in different species including *Saccharomyces cerevisiae* (Davis *et al*., 1992), *Arabidopsis thaliana* (Garcia-Hernandez *et al*., 1994), *Drosophila melanogaster* (Melnick *et al*., 1993), the sea urchin *Urechis caupo* (Rosenthal and Wordeman, 1995), *Chlorohydra viridissima* (Keppel and Schaller, 1991), the fungus *Candida albicans* (Lopez-Ribot *et al*., 1994) and the archaebacterium *Haloarcula marismortui* (Ouzonis *et al*., 1995), as well as in mammals (Ardini *et al*., 1998; for review: Rieger *et al*., 1999).The 37 kDa LRP also acts as a receptor for alphaviruses such as the Venezuelan equine encephalitis (VEE) virus on the surface of mosquito cells (Ludwig *et al*., 1996), has been identified on the cell surface of the fungus *Candida albicans* (Lopez-Ribot *et al*., 1994). and has been proved to be located on the surface of Madin-Darby canine kidney (MDCK) cells from dogs, which might be involved in cell attachment, spreading and polarization (Salas *et al*., 1992). These findings clearly demonstrate the location of the 37 kDa LRP on the cell surface.

Table II Characteristics of the 37 kDa laminin receptor precursor[a] (LRP)/67 kDa laminin receptor[b] (LR)

	Characteristics
Isolation	37 kDa LRP/p40 cDNA (Rao *et al*., 1989; Yow *et al*., 1988) 67 kDa LR isolated from solid tumors (Lesot *et al*., 1983; Malinoff and Wicha, 1983; Rao *et al*., 1983)
Occurrence of the 37 LRP/p40 gene	*Saccharomyces cerevisiae* (Davis *et al*., 1992), *Arabidopsis thaliana* (Garcia-Hernandez *et al*., 1994), *Drosophila melanogaster* (Melnick *et al*., 1993), *Urechis caupo* (Rosenthal and Wordeman, 1995), *Chlorohydra viridissima* (Keppel and Schaller, 1991), *Haloarcula marismortui* (Ouzonis *et al*., 1995), *Candida albicans* (Lopez-Ribot *et al*., 1994), mammals (Ardini *et al*., 1998)
Cellular localization of 37 kDa LRP	At the cell surface of mosquito cells (Ludwig *et al*., 1996) , of *Candida albicans* (Lopez-Ribot, 1994) and of mammalian cells such as Madin-Darby canine kidney cells (MDCK) (Salas *et al*., 1992); in the cytoplasm on 40S ribosomes (Auth and Brawerman, 1992); in the nucleus (Sato *et al*., 1996)
Molecular weight	37,000 (laminin receptor precursor protein) 67,000 (mature laminin receptor protein)
Binding partners of	
-37 kDa LRP	Laminin (Rieger *et al*., 1997), PrP^c (Rieger *et al*., 1997), the Venezuelan equine encephalitis (VEE) virus (Ludwig *et al*., 1996); association of LBP^c/p40 with histones H2A, H2B and H4 (Kinoshita *et al*., 1998)
-67 kDa LR	Laminin (Beck *et al*., 1990), elastin and carbohydrates (for review: (Ardini *et al*., 1998; Mecham, 1991; Rieger *et al*., 1999), the Sindbis virus (Wang *et al*., 1992)
Functional domains	Transmembrane domain: aa 86-101 (Castronovo *et al*., 1991b), Laminin binding domain: aa 161-180 (Castronovo *et al*., 1991b); PrP^c binding domain: aa 157 and 180 (Rieger *et al*., 1997)
Functions of	
- 37 kDa LRP	Receptor for laminin (Rieger *et al*., 1997), PrP^c (Rieger *et al*., 1997) and the Venezuelan equine encephalitis virus (Ludwig *et al*., 1996); as ribosomal protein LRP/p40 involved in protein synthesis (Auth and Brawerman, 1992); possible role of LBP^c/p40 in maintenance of nuclear structures (Kinoshita *et al*., 1998)
- 67 kDa LR	Receptor for laminin (Beck *et al*., 1990), elastin, carbohydrates (for review: (Ardini *et al*., 1997; Mecham, 1991; Rieger *et al*., 1997)) and the Sindbis virus (Wang *et al*., 1992); crucial role in the metastatic potential of solid tumors (Castronovo, 1991b)

[a] Laminin receptor precursor, LRP
[b] Laminin receptor, LR
[c] Laminin binding protein, LBP (equivalent to LRP)

Within the life cycle of prions, LRP may play a role in the physiological function of PrP^c, as well as in the pathogenesis of prion diseases. We assume that LRP is involved in the internalization process of PrP^c via cavolae-like domains (Vey *et al*., 1996) or clathrin-coated pits (Shyng *et al*., 1994) (Fig. 3). Involvement of clathrin-coated pits in the endocytosis of a

GPI-anchored protein such as PrP^c is surprising because PrP^c has no cytoplasmic domain that can interact directly with the intracellular components of coated pits (Harris, 1999). Here a receptor protein could be responsible for making the connection between the surface-anchored PrP to clathrin. The uptake of PrP^{Sc} is thought to be mediated directly by a receptor protein such as LRP, but could also be mediated in an indirect manner dependent on the presence of cellular PrP. We assume that internalized PrP^{Sc} interacts with PrP^c during the endocytic pathway (Fig. 3). PrP^c is probably converted into PrP^{Sc} within the endosome, lysosomes or endolysosome influenced by an unknown protein termed protein X (Telling *et al*., 1995) which could represent a molecular chaperone such as Hsp60 (Edenhofer *et al*., 1996). Recently, a homology of the amino terminus of LRP with members of the Hsp70 family was observed (Ardini *et al*., 1998) suggesting that LRP/p40 might be involved in protein folding. Although we demonstrated a specific interaction between PrP and members of the Hsp60 family including GroEL (Edenhofer *et al*., 1996), no binding of PrP to members of the Hsp70 family was observed, which suggest no homology to the Hsp60 family (Edenhofer *et al*., 1996). However, it cannot be excluded that a hypothetical chaperone activity of LRP might be involved in the PrP^c/PrP^{Sc} conversion reaction, which is thought to occur in endosomes, lysosomes or endolysosomes of the endocytic pathway in the life cycle of prions. Other proteins encompassing an GPI-anchor were internalized by caveolae (Anderson, 1993). It has been suggested that PrP^c and PrP^{Sc} are internalized by CLDs, a compartment where the conversion of PrP^c to PrP^{Sc} might also take place (Vey *et al*., 1996). PrP^{Sc} accumulation leads to neuronal cell death resulting in vacuolization and death of the organism.

The role of LRP within the life cycle of prions mediating PrP internalization and its involvement in pathological mechanisms within the complex scenario of transmissible spongiform encephalopathies has to be further investigated.

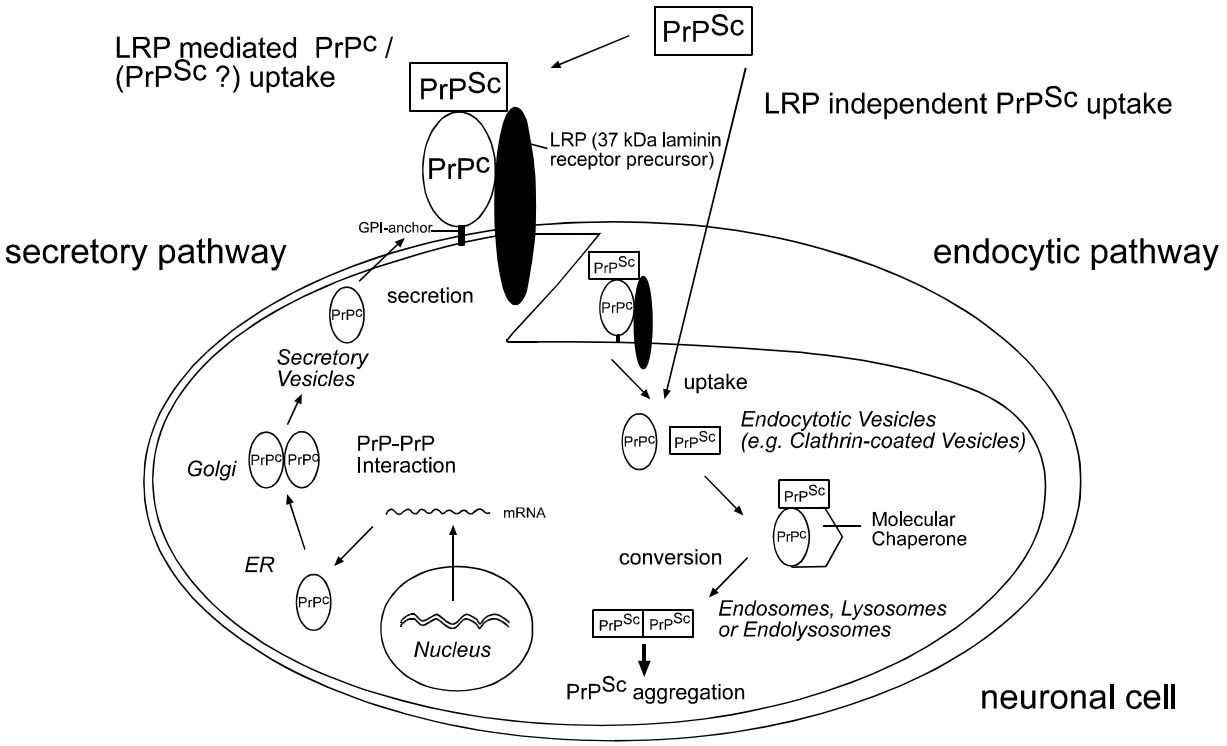

Fig. 3: Model of the life cycle of prions. PrP^c is synthesized in the rough endoplasmatic reticulum (ER), and after passing through the secretory pathway including the Golgi and secretory vesicles, reaches the surface of a PrP^{Sc} infectable cell where it is anchored via a glycosylphosphatidyl inositol (GPI) moiety. Endocytosis of PrP^c and possibly PrP^{Sc} via clathrin coated vesicles could be mediated by the 37 kDa laminin receptor precursor (LRP). The uptake of the infectious agent could also be LRP independent. The conversion of the internalized PrP^c to PrP^{Sc} is thought to take place in the endosomes, lysosomes or endolysosomes. Molecular chaperones could be involved in this conversion process. PrP replication and aggregation can occur in neuronal cells of the brain but also in the cells constituting the lymphoreticular system. Alternatively, endocytosis and conversion of PrP^c into PrP^{Sc} could happen in caveolae-like domains (CLDs).

D. The Cadherins

Two cell surface proteins were isolated from murine cells and characterized as so-called prion protein binding proteins (PrPBPs) (Table III) (Cashman and Dodelet, 1997). Mouse and human PrPs expressed as fusion proteins to human placental heat-stable alkaline phosphatase (PrP-AP) bound with high affinity to the surface of many primary cells and cell lines, particularly to the mouse muscle cell line G8, whereas no binding of AP alone could be observed. Frog oocytes showing little or no intrinsic PrP-AP surface binding were microinjected with *in vitro* transcribed mRNA generated from pooled plasmid clones of a G8

cDNA library. Following selection of clones that showed specific binding to PrP-AP, sequence analysis revealed the cDNA inserts in two clones, one encoded a portion of protocadherin-43 spanning amino acid residues 67 to 252 and exhibited the highest level of PrP-AP binding activity, the other one encoded a portion of OB-cadherin-1 (the N-terminal cadherin repeat) and showed a moderate PrP-AP binding (Cashman and Dodelet, 1997). Protocadherin-43 described by Sano *et al*. (1993) and OB-cadherin-1 described by Okazaki *et al*. (1994) belong to a group of cell adhesion proteins designated Cadherins. Cadherins are a family of transmembrane glycoproteins involved in Ca^{2+} dependent cell-cell adhesion that occurs in many tissues mediating development patterning and tissue organization. They contain a large N-terminal extracellular region consisting of repetitive subdomains including the Ca^{2+}-binding sites. Ca^{2+}-binding is required for cadherin interaction and cell-cell adhesion, a process that results from lateral clustering of cadherin *cis* dimers and their *trans* association with *cis* dimers on the apposed cell (Steinberg and McNutt, 1999). The C-terminus consists of a transmembrane region and a highly conserved cytoplasmic domain, through which cadherins interact with intracellular adhesions proteins such as catenins and stabilize the internal structure of the cell.

Binding of PrP-AP to cultured cells was significantly reduced in the presence of the calcium chelator EDTA, indicating that for optimum binding, the presence of divalent cations such as Ca^{2+} might be required. Binding of mouse, human and bovine cellular PrP as well as PrP^{Sc} from BSE-affected brain to the candidate receptor was observed (Cashman *et al*., 1999).

Prion proteins could act as novel ligands for cadherin proteins. Cadherins participate in cell-layer segregation and morphogenesis in development, also in maintenance of cell-cell recognition in mature tissues, and may participate in disorders in which recognition is deficient, such as metastatic cancer. It is also possible that they are involved in muscle and immunological disorders as well as in neurodegenerative diseases such as TSEs (Cashman and Dodelet, 1997). The possible role of cadherins as cell surface receptors for prion proteins, however, has still to be confirmed.

III. Molecular Chaperones of Mammals

The crucial event in prion diseases involves the conformational change of the cellular form of the prion protein into the pathogenic isoform. This change causes a dramatic alteration within the structure. Structural variations of a protein often require a catalysing agent. Molecular chaperones are prominent candidates that could promote this reaction.

The protein-only hypothesis indicates that the scrapie form of the prion protein can promote the conversion of the cellular form. This leads to the conclusion that prions themselves can act as chaperones (Liautard, 1991). Thermokinetic analysis of protein folding shows that a misfolded chaperone gives rise to new misfolded chaperones, which fit very well to the protein-only hypothesis in which PrP^{Sc} triggers the formation of PrP^{Sc}.

Besides this theory, other proteins can act as promotors for the prion conversion reaction. In 1996 chemical reagents were investigated and were shown to affect formation and propagation of PrP^{Sc}. Cellular osmolytes and proteinaceous chaperones were tested in this context (Tatzelt *et al*., 1996b). Chaperones that can prevent the formation of PrP^{Sc} (Fig. 4) might act as powerful tools for the generation of anti-TSE therapeutics.

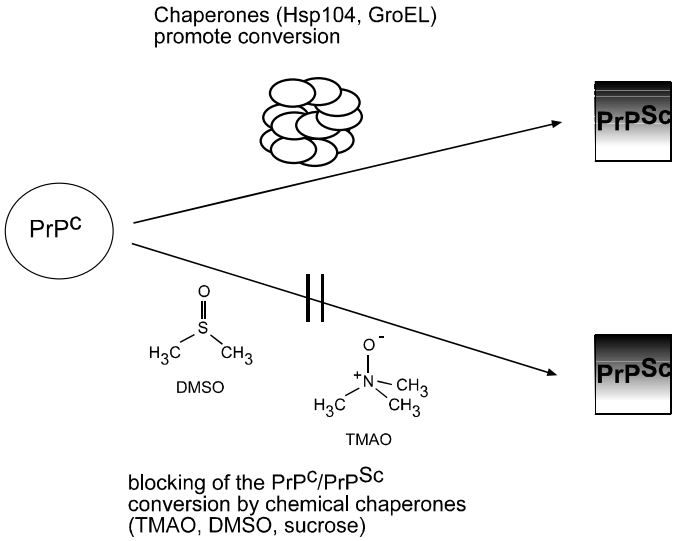

Fig. 4: Influence of molecular and chemical chaperones on the conversion process of PrP^{c} to PrP^{Sc}. Molecular chaperones such as Hsp104 and GroEL promote the conversion reaction whereas the chemical chaperones TMAO, DMSO and sucrose prevent PrP^{Sc} formation.

Molecular chaperones also represent a biochemical and mechanistical link between the mammalian prions and the "prion-like" proteins in yeast. In this light heat-shock protein Hsp104 has an effect on the conversion of hamster PrP (DebBurman *et al*., 1997) and on the regulation of the yeast nonchromosomal element [*PSI*+] (Chernoff *et al*., 1995) suggesting that the prion concept is of general importance in mammalian and nonmammalian systems.
Studies on the transmission of human prion proteins to transgenic mice indicates the existance of an unknown protein termed "protein X", which binds to PrP (Telling *et al*., 1995) and might act as a molecular chaperone.

A. Heat-Shock Proteins

A number of cellular proteins function *in vivo* as chaperones that catalyse the formation of proteins with an intact secondary, tertiary and quaternary structure. Heat shock proteins (Hsps) are prominent representatives of these chaperones and were first discovered because of their specific induction during the cellular response to heat shock (Gething and Sambrook, 1992). Nevertheless, the majority of the Hsps are expressed constitutively and their functions are diverse. Hsps stabilize unfolded protein precursors, rearrange protein oligomers and dissolve protein aggregates in an ATP-dependent manner.
Hsps are thought to play an important role in the conversion of the cellular prion protein PrP^{c} to the pathogenic isoform PrP^{Sc} (Table III). In 1995 the expression levels of Hsp72, Hsp28 and Hsp73 in normal and scrapie-infected mouse neuroblastoma cells were investigated (Tatzelt *et al*., 1995). After heat shock Hsp72 and Hsp28 were both detectable in normal, but not in scrapie-infected cells. The constitutively expressed Hsp73, however, was expressed at comparable levels in both cell types, indicating that Hsp73 could possibly assist the formation of PrP^{Sc}. The lack of Hsp72 and Hsp28 in scrapie-infected cells suggests that chaperones do not catalyse a refolding of PrP^{Sc} into PrP^{c} in these cells. Together, both facts might lead to an increase of PrP^{Sc} concentrations in scrapie-infected cells.
We identified Hsp60 as a PrP binding molecule employing a HeLa cDNA library in prey and hamster PrP in bait position of the yeast-two-hybrid system (Edenhofer *et al*., 1996). *In vitro* binding studies with recombinant PrP confirmed the specificity of the PrP-Hsp60 interaction. Mapping analysis employing a series of PrP peptides identified the C-terminus of PrP (aa 180 to aa 210) encompassing α-helix 2 and parts of α-helix 3 (179-193 and 200-217) (Riek *et al*., 1996; Donne *et al*., 1997; Riek *et al*., 1997) as the Hsp60 binding domain on PrP. GroEL, the prokaryotic homolog of Hsp60 revealed the same binding domain as Hsp60 on PrP. This

indicates that eukaryotic as well as prokaryotic chaperones interact with the prion protein and suggest an important role of heat shock proteins in the conversion process of prion proteins.

Table III Function of heat shock proteins and their effect on the prion protein

Heat shock protein	Reference	First reported function in prion diseases	Effect on PrP conversion
Hsp28	Tatzelt *et al*., (1995)	Role in Ca^{2+}-dependent thermoresistance	No effect on PrP conversion/PrP^{Sc} diminishes synthesis of Hsp28
Hsp40	DebBurman *et al*., (1997)	Co-chaperone of Hsp70s	No effect on PrP conversion
Hsp60	Edenhofer *et al*., (1996)	Stabilization of prefolded structures and folding	Binding to haPrP, binding domain: aa 180-210
Hsp70	DebBurman *et al*., (1997)	Completion of translocation in mitochondria	No influence on PrP conversion
Hsp72	Tatzelt *et al*., (1995)	Prevents aggregation and accelerates refolding of damaged proteins	No effect on PrP conversion/PrP^{Sc} diminishes synthesis of Hsp72
Hsp73	Tatzelt *et al*., (1995)	Cytosolic heat shock protein	Assists PrP^{Sc} formation?
Hsp90	DebBurman *et al*., (1997)	Stabilizing of inactive precursor forms in the cytosol	No influence on PrP conversion
Hsp104	DebBurman *et al*., (1997)	Thermotolerance and ethanol tolerance in yeast	Promotes conversion of PrP^{c}
GroEL	Edenhofer *et al*., (1996) and DebBurman *et al*., (1997)	Antifolding before translocation	Binding to haPrP, binding domain: aa 180-210, promote conversion of PrP^{c}
GroES	DebBurman *et al*., (1997)	Form functional complex with GroEL	No influence on PrP conversion

GroEL and the heat shock protein Hsp104 are able to affect the *in vitro* conversion of hamster PrP, confirming the importance of GroEL for the PrP conversion reaction (DebBurman *et al*., 1997). However, this process requires the presence of exogenous added PrP^{Sc}, suggesting that the conversion process and further aggregation seem to require a nucleation seed. Molecular chaperones may probably be not sufficient for this reaction. Other heat shock proteins like GroES, Hsp40, Hsp70 and Hsp90 do not show any effect in the conversion process. Hsp104 links mammalian prion proteins and the prion-like yeast protein Sup35. Hsp104 could therby either promote sup35* or sup35 formation dependening on Hsp104 concentrations. Hsp104

might influence the regulating process of the [PSI+] element in *S.cerevisiae* (Patino *et al*., 1996). In conclusion, heat shock proteins might influence the structure of mammalian and yeast prions.

B. Protein X

The transmission of human prion proteins to transgenic mice depends on the species of the endogenous expressed transgenic prion protein and the homozygocity/heterocygocity status of the expressed transgene. In contrast to transgenic mice ablated for the mouse *Prnp* gene or transgenic mice expressing low levels of a chimeric transgene, which are susceptible towards human prions, transgenic mice expressing the human PrP transgene are completely resistant towards human prions. This phenomenon reflecting the species barrier can be explained by a species specific factor termed protein X, which is thought to participate in prion formation. Protein X might act as a chaperone facilitating or hampering the conversion of PrP^{c} to PrP^{Sc}. The fact that transgenic mice hyperexpressing human PrP are resistant to human prions (Telling *et al*., 1995), together with the finding that transgenic mice expressing chimeric $MHu2MPrP^{c}$ retain human PrP susceptibility suggests that protein X could bind to the cellular form of the prion protein and the affinity of protein X to prion proteins of different species may vary. The binding of protein X to the prion protein may result in the PrP conversion reaction. Differences in the amino acid sequence of PrP of different species may be the main reason for both effects. The main differences between mouse and human PrP are thought to reside in the carboxy-terminus of PrP. An epitope mapping of the binding site for protein X on PrP (Kaneko *et al*., 1997b) by substitution of the basic residues at aa position 167, 171 or 218 preventing PrP^{Sc} formation suggests that the binding site for protein X on PrP resides within this region. Amino acid 218 is located within the third α-helix of the mouse prion protein and residues 167 and 171 reside within an adjacent loop. The stoichiometry of the protein X/ PrP^{c} complex is unknown to date. The fact that the protein X/PrP^{c} interaction was abolished by mutations preventing the PrP^{Sc} formation might be useful for the development of anti-TSE therapeutic agents. A prerequiste for that, however, is the identification of protein X.

C. Chemical Chaperones

In contrast to “classical” chaperones consisting of proteins, chemical chaperones represent chemical compounds of small molecular weight that are able to stabilize proteins and correct misfolded ones (Welch and Brown, 1996) (Fig. 4). Chemical chaperones such as glycerol, trimethylamine-N-oxide (TMAO) and dimethylsulfoxide (DMSO) might stabilize the native

conformation of a protein by direct interaction. These compounds termed „cellular osmolytes“ are produced in cells in response to osmotic shock (Somero, 1986). Glycerol, TMAO and DMSO were tested to determine their influence on the formation of PrP^{Sc} in ScN2a cells (Tatzelt *et al*., 1996b). All reduced the extent of PrP conversion into its detergent insoluble form. The stabilizing effect of the native form of a protein was also demonstrated for other proteins such as the cystic fibrosis transmembrane regulator (CFTR) (Brown *et al*., 1996). The presence of chemical chaperones might have an effect on the hydration of proteins. Because self-association or tighter packaging of the prion protein is enhanced, PrP^{Sc} fails to interact with PrP^{c} so that no PrP^{c}/PrP^{Sc} heterodimer is formed leading to an inhibition of the PrP conversion process (Gekko and Timasheff, 1981). In the case that chemical chaperones might be transported to the brain bypassing the blood-brain barrier (BBB), they might be useful as therapeutic agents in TSE-therapy.

The influence of chemical chaperones has also been demonstrated in cell-free conversion assays (DebBurman *et al*., 1997). The conversion of hamster PrP using partially denatured PrP^{Sc} was only inhibited by DMSO. Glycerol and cyclodextrin compounds had no effect, whereas molecular chaperones (Hsp104) were able to block the conversion process. Chemical chaperones such as glycerol and cyclodextrin, acting as co-chaperones, might have an influence on molecular chaperones that are lacking in a cell-free system.

IV. Interaction between prion proteins

According to the protein-only hypothesis, proposed by Prusiner (Fig. 5) the interaction of the cellular prion protein with the pathological isoform seems to be the crucial step in the conversion of PrP^{c} to PrP^{Sc}. The existence of the hypothetical PrP^{c}/PrP^{Sc} heterodimer may require the presence of a homodimer consisting of two PrP^{c} molecules. This homodimer is thought to be in equilibrium with the PrP^{c} monomers. It is unclear to date whether the spontanous conversion reaction involves PrP^{c} monomers or the PrP^{c} homodimers.

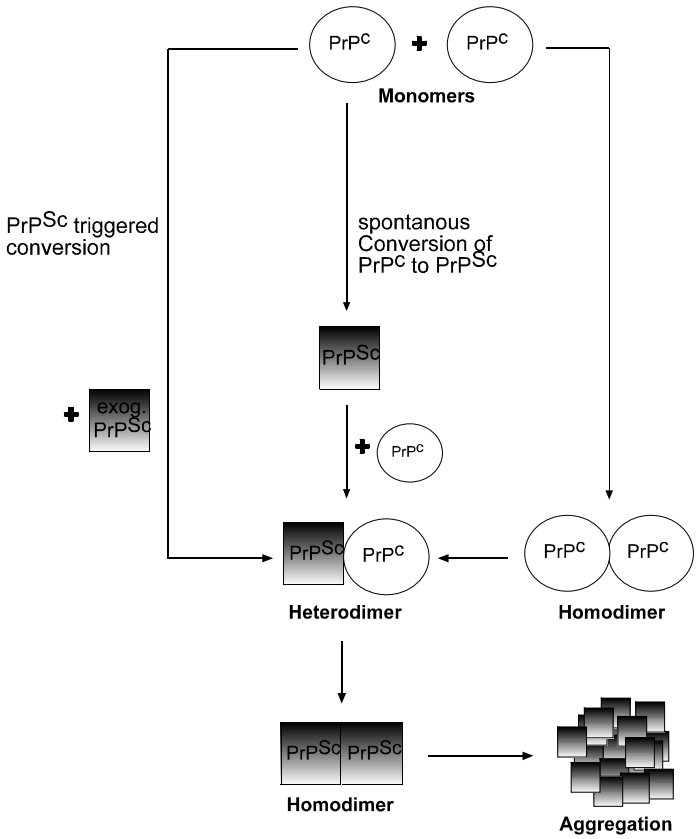

Fig. 5: Scheme of the conversion process of PrP^c to PrP^{Sc}. Three possibilities for the conversion of PrP^c into PrP^{Sc} do exist. An exogenous PrP^{Sc} triggers the conversion of PrP monomers leading directly to the hypothesized heterodimer consisiting of PrP^c and PrP^{Sc}. Genetic predisposition of an individual leads to a spontanous conversion of PrP^c to PrP^{Sc}. The conversion process might proceed after formation of a dimeric PrP^c or might occur with a monomeric PrP^c. The central PrP^{Sc} heterodimer forms a PrP^{Sc} homodimer aggregating into amyloid fibrils.

In 1986 a 54 kDa protein was identified under denaturing conditions that may act as a dimeric PrP precursor for the scrapie protein (Bendheim and Bolton, 1986). A 60 kDa form of a recombinant hamster prion protein was detected in murine neuroblastoma cells in 1995 (Priola *et al*., 1995). It appears as a dimer under denaturing conditions analyzed by SDS-PAGE and under native conditions analyzed by immunoprecipitation. The linkage of both prion proteins might occur via hydrogen bonding, electrostatic interactions or covalent linkage involving lysins at the N-terminus of the protein. The observed dimer formation might be due to the hyperexpression of PrP with high PrP concentrations.

The multimer formation of the prion protein and structural changes during this process has been investigated by fluorescence correlation spectroscopy (FCS) (Post *et al*., 1998). Prion aggregates mainly constituted of PrP27-30 were converted by sonication to monomeric PrP

with an high α-helical content in the presence of 0.2% SDS. The oligomerization process was then initiated by the reduction of the SDS-concentration. Formation of β-sheet structured dimers was the initial step followed by oligomerization of these dimers within 10 minutes. After 1 hour PrP was aggregated. Whether the conversion reaction arises before the dimerization event or wether dimerization represents the initial step of the conversion process remains speculative.

Prion proteins with mutations in the octarepeat region causing familial CJD show abnormal aggregation properties (Priola and Chesebro, 1998). Hamster PrPs encompassing two, four and six octarepeats were expressed in mouse neuroblastoma cells. The fact that PrP dimers were detectable even under harsh denaturing conditions present in SDS-gel electrophoresis suggest that the PrP monomers were covalently linked rather than stabilized by noncovalent linkages such as hydrophobic interactions. However, covalently linked PrP dimers have still to be confirmed by other systems.

Because of the lack of convincing experimental data, only a few models describe the PrP-dimerization process. One of them proposes the highly conserved region from aa 109 to aa122 as a major dimerization domain (Warwicker and Gane, 1996) calculated by a computational search for potential PrP interaction interfaces. Mutations such as alanine to valine at position 117 of human PrP associated with Gerstmann-Sträussler-Scheinker syndrome reside within this region, and might alter the stability of the dimer, facilitating the conversion of PrP^{c} to PrP^{Sc}. In addition to the dimerization process, the association of the prion protein to the membrane could play an important role in TSE pathogenesis (Warwicker, 1999). The putative membrane-binding domain might be the first α-helix. The agglomeration of the prion protein on the membrane might influence the orientation and configuration of PrP facilitating the PrP interaction process.

Whether PrP dimers that have also been observed by us (Hundt, Gauczynski, Riley, and Weiss, manuscript in preparation) might play an important role in the PrP oligo-/multimerization process and whether PrP/PrP interfering agents might hamper the entire PrP aggregation process have still to be investigated.

V. Other PrP interacting molecules

This section first describes PrP interacting molecules identified by ligand blots, yeast two-hybrid techniques or *in vitro* selection. Members of the PrP ligand family Pli are described followed by Bcl-2 belonging to the family of proapoptotic and antiapoptotic molecules. Second, molecules are summarized acting as therapeutics in TSEs. With the exception of the protein clusterin, all the other molecules are of nonproteinaceous origin including polyanions, Congo red, polyene antibiotics, IDX, porphorins and phtalocyanes. Finally, nucleic acids such as RNA aptamers are described in their function as PrP-interacting molecules.

A. PrP Ligands (Pli´s)

1. Pli 45 and Pli 110

Two PrP binding proteins were identified in 1990, using ligand blots (Oesch *et al*., 1990). These two proteins identified from hamster brain were termed PrP ligands Pli 45 and Pli 110. To investigate the interaction of purified PrP with other proteins the authors used radiolabeled PrP27-30 and PrP^{c}, respectively, for the binding of proteins from hamster brain that were separated by SDS-PAGE and blotted to nitrocellulose (ligand blots).Two major bands became visible by autoradiography using purified PrP27-30 and immunopurified PrP^{c}. The molecular weight of the identified proteins were 45,000 and 110,000, respectively, and both proteins bound to PrP^{Sc} and PrP^{c} derived from hamster brain. Other PrP binding proteins ranging from 32-200 kDa were also observed. The stability of the complexes formed by Pli 45 and PrP 27-30 on nitrocellulose were investigated by intense washing steps and 50% of the radiolabelled PrP27-30 was washed off after 60 hours, corresponding to a dissociation rate constant of $k_D=3x10^{-6}$ s^{-1}. Pli 45 revealed a sequence homology of 94.6% to murine GFAP (glial fibrillary acidic protein) at the cDNA level, suggesting that Pli 45 and GFAP are the same proteins. Comparitive immunochemistry studies, using polyclonal Pli45- and GFAP specific antibodies revealed the same staining pattern as monoclonal anti-GFAP antibodies in scrapie-infected sheep brain. In addition, both antibodies recognized recombinant GFAP expressed in *Escherichia coli*, suggesting that Pli 45 and GFAP are indeed the same proteins.

Pli 45 was found exclusively in brain, whereas Pli 110 is present in several tissues, such as brain, lung, liver, spleen and pancreas. Pli 110 was shown to be identical with PTP-associated splicing factor (PSF) (Oesch, 1994). Because studies with $GFAP^{0/0}$ mice revealed that GFAP is not essential for scrapie development (Gomi *et al*., 1995; Tatzelt *et al*., 1996) and PSF is an

essential splicing factor, located in the nucleus (Patton *et al*., 1993), it seems that Pli45 and Pli110 do not play a crucial role in prion diseases.

2. Pli3-Pli8

Seven years after the identification of the first two PrP-binding proteins Pli 45 and Pli 110 six other PrP ligands were found (Table I) (Yehiely *et al*., 1997). The authors used a different system than that used for the identification of Pli 45 and Pli 110. Here, PrP was designed as a fusion protein with alkaline phosphatase (AP) and secreted by NIH 3T3 cells. PrP-AP was then used as a probe for screening the mouse brain cDNA library λgt11. Sequence analysis of nine clones revealed the six unique sequences, *Pli3* to *Pli8*. Two cDNA clones showed homology to known sequences, to the mouse amyloid precursor-like protein (*Aplp1*) denoted Pli6 and to the mouse p45 NF-E2 related factor 2 (*Nrf2*), termed Pli7. All six Plis revealed the consensus sequence GXXXXXX(E/P)XP, which is not unique to PrP binding proteins, but was identified in many other protein sequences. Hence, the authors conclude that it might represent a functional motif. Negative charge might also play a role in PrP binding, as four cDNA clones showed an excess of glutamic acids and aspartic acids over lysines and arginines. Each cDNA clone identified a single copy gene and the chromosomal location of each clone was identified in this work.

Polyclonal antibodies directed against the polypeptides Pli3 and Pli5 were generated and purified. Both antibodies recognized proteins from N2a cells and mouse brain on Western blots. Anti-Pli3 antiserum detected a 70 and a 100 kDa polypeptide, whereas anti-Pli5 antiserum detected a 45 kDa polypeptide. All three identified polypeptides were believed to be novel PrP-binding proteins. Antisera to Nrf2 (Pli7) and Aplp1 (Pli6) were also used as probes on N2a cell lysates and mouse brain homogenates. For anti-Nrf2 antiserum, a 66 kDa protein was found, that corresponds to the predicted size of mouse Nrf2. Aplp1 antiserum recognized polypeptides of about 85 and 95 kDa molecular mass, which are likely to be two different forms of Aplp1. The protein levels of Pli3 and Pli5 appeared similar in scrapie-infected and noninfected brain and N2a cells, whereas higher levels of Pli5 mRNA could be found in ScN2a cells. The protein levels of Nrf2 were found to be slightly decreased in ScN2a cells, whereas Aplp1 protein levels remained unchanged in ScN2a cells and infected mouse brain. Higher mRNA levels for both Aplp1 and Pli5 were found in ScN2a cells.

Aplp2 is a member of the APP-like (amyloid precursor protein) family, playing an important role in the pathogenesis of Alzheimer disease (AD). The major component of the senile plaques that are observed in AD is the Aβ peptide, which is derived from the APP protein

(Glenner and Wong, 1984; Masters *et al*., 1985). PrP and Aplp1 are both membrane proteins; hence it is likely that they could interact on the cell surface.

B. Bcl-2

Bcl-2 (Table I) represents a well-known member of a rapidly enlarging protein family of proapoptotic and antiapoptotic molecules, including at least 15 related proteins (Adams and Cory, 1998). In 1995 the role of Bcl-2 was investigated using a yeast two-hybrid screen (Kurschner and Morgan, 1995). LexA-Bcl-2 in the bait and a murine cerebellar cDNA-VP16 fusion library in the prey position identified potential Bcl-2 binding proteins. Surprisingly the prion protein and not bax, which is known to heterodimerize with Bcl-2 (Oltvai *et al*., 1993), was pulled out by this screen. The sequenced cDNA clone contained a fusion between the VP16 domain and mouse PrP, encompassing aa72 to aa245, denoted PrP-VP16. Usinging LexA-PrP in the bait and Bcl-2-VP16 in the prey position of the yeast two-hybrid system resulted also in an interaction between PrP and Bcl-2. Interactions with other members of the Bcl-2 family, such as Bax or A1 were not observed. The PrP mutation P102L, associated with human Gerstmann-Sträusler-Scheinker syndrome was investigated, and it was shown that this mutation did not alter the binding behavior of PrP to Bcl-2. Interestingly, the PrP-Bcl-2 interaction could not be confirmed by coimmunoprecipitation assays, suggesting that this protein interaction can be observed only in the yeast-two-hybrid system.

Bcl-2 and Bax act as antiapoptotic and proapoptotic molecules in apoptosis, respectively. Moreover, the ratio of Bax-Bcl-2 heterodimers to homodimers of each protein is important for the regulation of apoptosis (Oltvai and Korsmeyer, 1994; O´Dowd *et al*., 1988; Yang and Korsmeyer, 1996). Hence the authors concluded that PrP might play a role in disrupting the Bax:Bcl-2 ratio by trapping Bcl-2 and favoring Bax-Bax homodimers, which would lead to cell death by apoptosis (Fig.6). The trapping of Bcl-2 by PrP might occur during trafficking of PrP before exposure to the cell membrane. Although Bcl-2 and PrP are both membrane associated, the physiological cellular location of Bcl-2 is different from that of PrP. Bcl-2 is thought to be an inner mitochondrial membrane protein (Hockenbery *et al*., 1990; Motoyama *et al*., 1998) or might reside on the mitochondrial outer membrane, the endoplasmatic reticulum, or the nuclear membrane (Krajewski *et al*., 1993; Lithgow *et al*., 1994) , and is not present on the cell surface membrane.

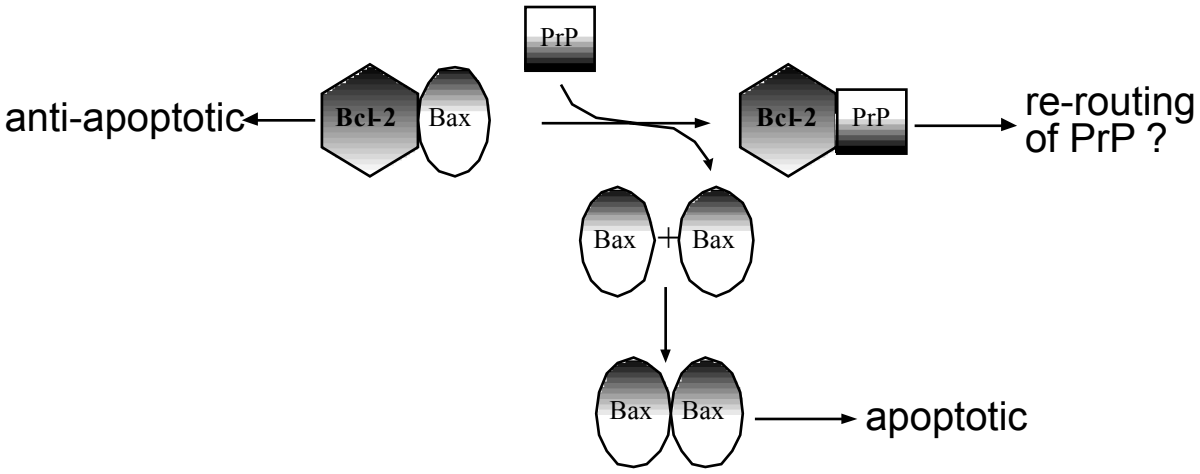

Fig. 6: Schematic view of Bcl-2::Bax, Bcl-2::PrP and Bax::Bax transitions and their possible role in cellular functions.

C. Laminin

Laminin (LN) is a glycoprotein of the extracellular matrix (ECM) [for review see Beck *et al*., (1990)] that mediates cell attachment, communication, differentiation, movement and neurite outgrowth promotion (Hunter *et al*., 1989). Laminin is the first ECM protein detected during embryogenesis. In later development and in mature tissue, laminin serves as an ubiquitious and major noncollagenous component of basement membranes (Beck *et al*., 1990). Laminin was first isolated from Engelbreth-Holm-Swarm (EHS) tumor (Timpl *et al*., 1979) and from extracellular deposits of murine parietal yolk sac (PYS) carcinoma cells (Chung *et al*., 1979). A specific binding between laminin and the amyloid precursor protein (APP), the precursor of the amyloid peptide involved in Alzheimer`s disease, has been identified (Narindrasorasak *et al*., 1992). APP and β-amyloid peptide (1-40) interaction with the extracellular matrix promotes neurite outgrowth, suggesting that the complex might play a normal physiological role in the brain (Kibbey *et al*., 1993; Koo *et al*., 1993). Recently, a direct interaction between the cellular prion protein (PrP^c) and laminin was reported (Graner *et al*., 2000). An involvement of the PrP^c-laminin interaction in neuritogenesis induced by NGF plus laminin in the PC-12 cell line was further suggested (Graner *et al*., 2000). Neuritogenesis, induced either by laminin or its γ-1-derived peptide in primary cultures from rat or either wild-type or PrP null mice hippocampal neurons, might imply that PrP^c could be the main cellular receptor for the particular γ-1 domain located to the carboxy terminus of laminin (Graner *et al*., 2000).

D. Therapeutics

1. Polyanions

Polyanions (Table IV), including heteropolyanion 23 (HPA-23), Dextran Sulfate 500 (DS 500), pentosan polysulfate (SP54) and heparin are known to bind the prion protein and/or prevent PrP^{Sc} accumulation in animals and cell systems (Brimacombe *et al*., 1999; Caughey and Raymond, 1993; Diringer and Ehlers, 1991; Ehlers and Diringer, 1984; Farquhar *et al*., 1999; Gabizon *et al*., 1993; Kimberlin and Walker, 1983; Kimberlin and Walker, 1986; Ladogana *et al*., 1992). The first polyanion denoted as an anti-scrapie drug was HPA-23 (Kimberlin and Walker, 1983; Kimberlin and Walker, 1986). The effect of HPA-23 was tested in several different scrapie strains, such as 139A, ME7, 22A and 263K. HPA-23 was effective in all these strains and prolonged the lifetimes of the animals significantly after scrapie injection. Less effect was observed when scrapie material was injected intraperitoneally or if the drug was given more than 48 hours after scrapie infection. Injection before to infection with scrapie is not effictive, owing to the rapid metabolization or excretion of HPA-23. HPA-23 is thought to interfere with early replication of PrP^{Sc} in the lymphoreticular system, reducing the efficiency of scrapie infection. These results, together with the brain toxicity of this molecule suggest, that HPA-23 has limited therapeutic value.

Two high-molecular-weight polyanions, carrageenan and DS 500, were shown to be highly efficient in reducing scrapie titers in mice infected with the 139A strain of scrapie (Ehlers and Diringer, 1984; Kimberlin and Walker, 1986). All intravenous or intraperitoneal combinations of injecting DS 500 or scrapie reduced the effective titer about 100- to 200 fold. The effect of DS 500 is long-lasting. Application of DS 500 up to 10 weeks before to infection increases the incubation period in mice. However, DS 500 itself is highly toxic and causes up to 50% mortality at a dose of 2 mg per mouse. Like HPA-23, DS 500 is thought to prevent PrP^{Sc} replication in spleen and lymph nodes and its mode of action is likely to be independent of its activity as a B-cell mitogen. The high-molecular-weight and negative charge may represent important factors in the anti-scrapie effect of DS 500. SP54 (Pentosan Polysulfate, Fig. 7A) has an anti-scrapie effect comparable to DS 500, but is less toxic. It has been shown that SP54 significally increases scrapie incubation period in hamsters infected with 263K scrapie strain and in mice infected with the 139A, Me7 and 22A strains of scrapie (Ehlers and Diringer, 1984; Farquhar *et al*., 1999; Ladogana *et al*., 1992). SP54 is even effective if only a single low-dose is injected after infection. A single injection of 250 μg of SP54 increased the mean incubation period of the ME7 strain by up to 66% and 1 mg of SP54 protected mice completely from the 22A scrapie strain. SP54 is thought be effective during the very early

events of pathogenesis by interfering with the uptake of PrP^{Sc} by nerve endings and/or carrier cells. The low-dose effect and the lower *in vivo* toxicity compared to other polyanions make SP54 a promising candidate in the field of anti-scrapie polyanions.

All anti-scrapie polyanions published so far might act by competing directly with the binding of cellular glycoaminoglycans (GAGs) to PrP^{c} (see chapter II. A) and/or PrP^{Sc} (Brimacombe *et al*., 1999; Caughey *et al*., 1994). Indeed, GAGs are involved in the metabolism of PrP^{c} (see chapter II.A) and thus in the biogenesis of PrP^{Sc}. It was shown by surface plasmon resonance, that pentosan polysulfate shows the strongest binding to recombinant PrP followed by heparin and dermatan sulfate. This correlates to the ability of the molecules to delay scrapie disease and reduce PrP^{Sc} accumulation in scrapie-infected cell lines (Caughey and Raymond, 1993).

2. Congo Red

Congo red (Fig. 7C, Table IV) is a dye that can be used as a diagnosic stain for amyloids. It is well known that Congo red can inhibit PrPres accumulation in Sc^{+}-MNB cells and PrP^{Sc} replication in 263K and 139H treated hamsters (Caspi *et al*., 1998; Caughey *et al*., 1994; Caughey *et al*., 1993; Ingrosso *et al*., 1995). The mechanism of the Congo red anti-scrapie effect probably involves direct binding to PrP^{c}, which again is thought to block the binding of cellular GAGs to PrP^{c}, as described for polyanions (Caughey *et al*., 1994). The proposed direct binding of Congo red to PrP^{Sc} is thought to stabilize PrP^{Sc}, the abnormal isoform of the prion protein, and prevents its partial denaturation, which could be necessary for agent replication (Caspi *et al*., 1998).

3. Polyene Antibiotics

Amphotericin B (AmB) and MS-8209 (Fig. 7D) are polyene macrolide antibiotics, that have a ring structure containing a hydrophobic and a hydrophilic region on either side of the molecule. They are used for the treatment of systemic fungal infections like candidiasis, histoplasmosis and aspergillosis (Medoff *et al*., 1983). The effects of AmB and its derivative MS-8209 were studied in several models of rodents including 263K-infected hamsters. Both were very efficient in delaying scrapie disease and PrP^{Sc} accumulation. MS-8209 shows at least a five times lower toxicity and a higher solubility and is able to double the incubation time of scrapie in hamsters. In contrast to polyanions, polyene antibiotics are effective even after intracerebral infection (Adjou *et al*., 1995; Demaimay *et al*., 1994; McKenzie *et al*., 1994; Pocchiari *et al*., 1987; Xi *et al*., 1992).

Table IV. Antiscrapie drugs likely to interact directly with PrP

Drug	Tested scrapie strain	Successfully treated animals	Suggested mode of action	Comments	References
HPA-23	139A, ME7, 22A and 263K	Mouse and hamster	Prevents early agent replication in the LRS, competes with GAG (glycosaminoglycan) binding site	Effective in a lot of scrapie strains, rapid metabolism and excretion, toxic	Kimberlin and Walker (1983; Kimberlin and Walker (1986
DS 500	139A	Mouse	Prevents agent replication in the LRS due to its high molecular weight and negative charge, competes with GAG (glycosaminoglycan) binding site	Long-lasting anti-scrapie effect but toxic at therapeutic doses	Ehlers and Diringer (1984); Kimberlin and Walker (1986)
Pentosan Polysulfate	139A, ME7, 22A and 263K	Mouse and hamster	Interferes with PrP^{Sc} uptake from nerve endings, competes with GAG (glycosaminoglycan) binding site	Very promising drug, effective at extreme low dose	Ehlers and Diringer (1984); Farquhar *et al*. (1999); Ladogana *et al*. (1992)
Amphotericin B	C506M3 and 263K	Mouse and hamster	Direct prevention of PrP conversion or interference with PrP^{Sc} uptake	Acute nephrotoxicity and low solubility, widely used for the treatment of fungals	Pocchiari *et al*. (1987); Xi *et al*. (1992)
MS-8209	C506M3 and 263K	Mouse and hamster	Same as for AmB	Lower toxicity than AmB	Adjou *et al*. (1995); Demaimay *et al*. (1997) Adjou *et al*. (1999)
Congo Red	263K and 139A	Hamster	Binding to PrP^{c} with polyanion-like behavior, or binding to PrP^{Sc} (overstabilisation)	Dyes amyloid	Caspi *et al*. (1998); Caughey *et al*. (1993); Ingrosso *et al*. (1995)
Anthrycycline	263K	Hamster	Binding to PrP^{Sc}, preventing amyloid	Used for the treatment of malignancies	Tagliavini *et al*. (1997)

			deposition		
Porphyrins and Phtalocyans	263K	Mouse expressing hamster PrP	Binding to PrP^{Sc}	Inhibits cell free $PrP^{c/Sc}$ conversion	Caughey *et al*. (1998); Priola *et al*. (2000)
Cp-60/Cp-62	ScN2a cells	None	Mimicking dominant negative inhibition of prion replication	Identified by using a computational database search	Perrier *et al*. (2000)
IPrP13 (β-sheet breaker)	139A	Mouse	Direct change of PrP secondary structure	Synthetic peptide	Soto *et al*. (2000)
Clusterin [apolipoprotein J (apo J)]	-----	None, prevents aggregation of PrP106-126	Binding to $PrP^{c/Sc}$	Binds to extraneuronal PrP^{BSE}	McHattie and Edington (1999)

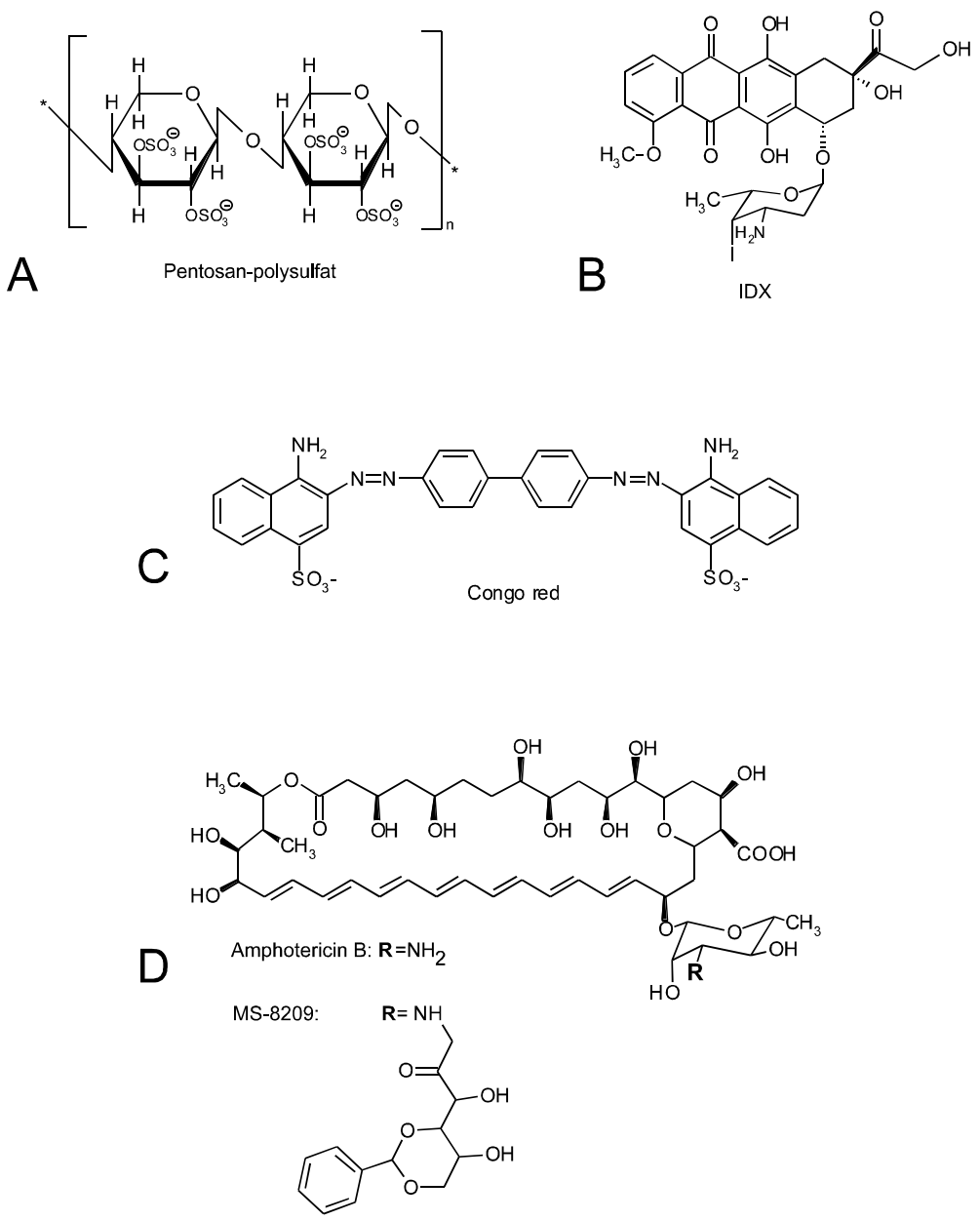

Fig. 7: Antiscrapie drugs of four different classes. (A) Pentosan polysulfate as a powerful drug belonging to the polyanion family. (B) IDX a derivative of doxorubicin. (C) Congo red belonging to the diazo dyes. (D) Amphotericin B and MS-8209 belonging to the family of polyene macrolide antibiotics.

Presently AmB and its derivatives are the only category of antiscrapie drugs that are prolonging the incubation period when given at late stages of infection (Demaimay *et al*., 1997). However, the effect of polyene antibiotics vary between scrapie strains (Adjou *et al*.,

1996). Note that the only reported treatment of clinical CJD with AmB in humans was unsuccessful (Masullo *et al*., 1992). Several possible mechanisms are involved in the antiscrapie effect of polyene antibiotics. AmB and MS-8209 have been proposed to directly affect the PrPsen to PrPres conversion step and thus prevent PrPres accumulation (Adjou *et al*., 1999; Adjou *et al*., 1997; Demaimay *et al*., 1997). Nevertheless a more indirect mode of action seems to be possible, whereby AmB and its derivatives disturb the uptake of PrPres by cells most likely by interfering with membrane cholesterol-rich domains (rafts) (Bolard, 1986; Taraboulos *et al*., 1995).

4. Other Therapeutics

Anthracycline 4′-iodo-4′deoxy-doxorubicin (IDX) (Fig. 7B; Table IV) is a derivative of the drug doxorubicin, which is successfully used in the treatment of several malignancies (Barbieri *et al*., 1987). IDX binds to amyloid fibrils and induces amyloid resorption in patients suffering from plasma cell dyscrasias with immunoglobulin light-chain amyloidosis (Gianni *et al*., 1995; Merlini *et al*., 1995). IDX was shown to delay the clinical signs of scrapie disease in 263K-infected hamsters when co-incubated with the 263K material prior to intracerebral inoculation. At a molecular level IDX is thought to bind the abnormal form of PrP, thereby decreasing the number of template molecules available for the PrP^c conversion process (Tagliavini *et al*., 1997).

Porphyrins and phtalocyans (Table IV) prevented PrPres accumulation in scrapie-infected mouse neuroblastoma cell cultures (Caughey *et al*., 1998) and prolonged the incubation period in hamster PrP expressing mice infected with 263K scrapie (Priola *et al*., 2000). The molecules also inhibited a cell-free conversion of hamster PrPsen to PrPres, showing that the effect seems to be due to direct PrP-binding. Nevertheless, because PrPres preparations are not completely pure, interactions with other molecules might be possible. Some other interactions with cells involved in scrapie pathogenesis can also not be excluded (Manuelidis, 2000).

Based on the proposal of a protein X binding domain (Kaneko *et al*., 1997b) synthetic drugs were identified that are able to inhibit PrP^{Sc} formation in ScN2a cells (Perrier *et al*., 2000). Two compounds, Cp-60 and Cp-62 (Table IV) act in a dose-dependent manner and show low toxicity. They are suggested to mimic the dominant negative inhibition of PrP replication originally reported for a PrP mutant (Kaneko *et al*., 1997a).

A 13-residue β-sheet breaker peptide (iPrP13) (Table IV) was shown to partly reverse PrP^{Sc} to a PrP^c like state. Mice inoculated with iPrP12-pretreated infectious material showed delayed appearance of clinical symptoms (Soto *et al*., 2000). The peptide is thought to directly change

the conformation of PrP^{Sc} from a β-sheeted to a more α-helical secondary structure and therefore reduce infectivity.

An effect of clusterin (Table IV) on the *in vitro* aggregation of the prion neuropeptide 106-126 was tested. Clusterin co-localizes with extraneuronal PrP^{BSE} in terminal BSE and the aggregation of the neuropeptide 106-126 was inhibited by clusterin in a dose-dependent manner (McHattie and Edington, 1999). The neurotoxicty of peptide 106-126 is subject of discussion, since a recent report described aggregation but no neurotoxicity for this peptide (Kunz *et al*., 1999).

Dapsone (Manuelidis *et al*., 1998) and flurpirtine (Perovic *et al*., 1995) have also been described as TSE therapeutics. In contrast to the previously described drugs, however, a direct interaction with PrP is unlikely (Table V).

Table V. Antiscrapie drugs not thought to interact directly with PrP

Drug	Tested on scrapie strain	Success in animal treatment	Suggested mode of action	References
Dapsone	SY	Mouse	Altering of macrophage processing of infectious agent and modulation of inflammatory factors	Manuelidis *et al*, (1998)
Flurpirtine (Katadolon)	----	None, cures neuronal cells treated with PrP106-126	Lowers toxic effect of PrP106-126 by normalization of GSH levels	Perovic *et al*. (1995)

E. Nucleic Acids

So far, no nucleic acid directly linked to scrapie infectivity has been identified. The existence of scrapie-specific homogeneous nucleic acid of more than 80 nucleotides has been excluded by analysis of highly purified scrapie preparations involving improved return refocusing gel electrophoresis (Kellings *et al*., 1992). However, the presence of a nucleic acid associated with infectivity cannot be ruled out, as the BSE agent can be transmitted to mice in the absence of detectable abnormal PrP (Lasmézas *et al*., 1997).

The *in vitro* interaction of nucleic acid with PrP has been described for both DNA and RNA. Using fluorescence labelled DNA, it was shown that the bindingstrength of peptide PrP106-126 to DNA was of a similar order of magnitude as the binding of retroviral protein p10 with model nucleic acids (Nandi, 1997). It was also shown that PrP106-126 polymerizes in the presence of DNA in solution, whereas the peptide alone fail to polymerize (Nandi, 1998).

RNA aptamers that bind specifically to recombinant hamster PrP (Weiss *et al*., 1995) but not to recombinant PrP90-231 (Weiss *et al*., 1996) were isolated by *in vitro* selection (Weiss *et al*., 1997). RNA aptamers of three different motifs were isolated, and all revealed a G quartet scaffold, which was proved to be essential for PrP^{c} binding. An RNA aptamer of only 29 nucleotides, representing the G quartet scaffold, was sufficient for PrP^{c} recognition. The interaction of the G quartet scaffold with PrP^{c} was directed exclusively against the amino terminus (aa23-52) of PrP. However, it could not be excluded that the aptamer recognizes PrP^{Sc}, but failed to recognize PrP27-30, lacking aa23-89 from the amino terminus.

Chapter III

Intra- and interspecies interactions of prion proteins and effects of mutations and polymorphisms

submitted for publication:
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Abstract

Recently, crystallization of the prion protein in a dimeric form was reported. Here we show that native soluble homogeneous FLAG tagged prion proteins from hamster, man and cattle expressed in the baculovirus system are predominantly dimeric. The PrP/PrP interaction was confirmed in SFV-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 failure 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 system to investigate species barriers in prion diseases.

Introduction

Prions are thought to be the infectious agents of transmissible spongiform encephalopathies (TSEs) (for review, see Ref. (Lasmézas and Weiss, 2000; Prusiner *et al*., 1998; Weissmann and Aguzzi, 1997)). 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*., 1997b) and the 37 kDa/67 kDa laminin receptor (Rieger *et al*., 1997), very recently identified as the receptor for the cellular prion protein (Gauczynski *et al*., 2001b). Direct interactions of the prion protein with itself have not been investigated so far.

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) (Jansen *et al*., 2001; Post *et al*., 1998). PrP dimers have been observed in N_2a cells and in scrapie-infected hamster brains (Priola *et al*., 1995). Molecular modelling suggested the existance 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). Very recently, crystallization of dimeric PrP has been reported involving domain swapping of α-helical structures (Knaus *et al*., 2001). 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 carboxyterminus of the prion protein (PrP90-230) as PrP/PrP interaction domains.

Defined mutations within the *Prn*-p gene lead to familial Creutzfeldt Jakob Disease (fCJD), Gerstmann-Sträussler-Scheinker (GSS) Syndrom 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 102 proline 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). We investigated whether relevant mutations and polymorphisms within the *Prn*-p

gene affect the PrP/PrP interaction behaviour. 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 themselves. Point mutations which are located in the second PrP/PrP interaction domain (90-230), however, leading to amino acid substitutions at position 102 (proline to leucine), 129 (methionine to valine) and 178 (aspartate to asparagine), respectively, did not influence the PrP/PrP interaction behaviour 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, suggesting that this system might be a useful and rapid pre-assay system 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 weight of approx. 27 kDa under denaturing conditions on an SDS-PA-gel (Figure 1 A, D and G) and was recognized by PrP specific antibodies (Figure 1 B, E and H). Under native conditions, however, FLAG tagged hamster, bovine and human PrP revealed molecular weights of 53, 54 and 53 kDa, respectively, and to a minor extent molecular weights of 24, 25 and 24 kDa, respectively, as determined by size exclusion chromatography (Figure 1 C, 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 % wheras the proportion of the dimeric form decreased from 98.1 to 93.4 % (Figure 1 J), suggesting 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 1 K, L) was monomeric under native conditions as measured by size exclusion chromatography (Figure 1 M) demonstrating that the FLAG tag is not responsible for the dimerization behaviour of FLAG tagged PrP.

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 an eucaryotic system, we chosed 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 immobilizing 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 and PrP227-FLAG-228, 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 to the nickel-column due to the histidine-rich octa-repeat region of PrP (lane 4). 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) 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). The system confirms the PrP/PrP interaction in BHK cells.

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 position 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 failed to interact with itself and 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 (Donne *et al*., 1997; Riek *et al*., 1997) (also known as the proline/glycine rich region), 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 (PHGGGWGQ). 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 existance of a second PrP/PrP interaction domain. Co-expression of PrP90-230 in both bait

and prey position 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 behaviour. When human PrP encompassing two (Figure 4, row 7), five (row 8) and nine (row 9) additional octarepeats were expressed in bait versus wild-type PrP in 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 so far hypothetical case of a patient homozygous for this *Prn*-p mutation, the PrP/PrP interaction was in the case of two additional octarepeats strongly reduced (row 4) and in case of five (row 5) and nine (row 6) additional octarepeats completely abolished.

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 behaviour. Neither this polymorphism nor the mutation at position 102 expressed in bait position versus wild type PrP in the prey position (Figure 5, lanes 6 and 4, respectively) or both expressed in both bait and prey position (Figure 5, lanes 7 and 5, respectively) reflecting the heterozygous and homozygous state, respectively, affected the PrP/PrP interation behaviour. In addition, the FFI related mutation 178 Asp to Asn (aa129 methionine) did not influence the PrP/PrP interaction behaviour when expressed in bait versus wild-type PrP in 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 behaviour. 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 interact 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 all PrP species failed to interact with GST (Figure 6, row 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 process 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 weight 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.

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 (Meyer *et al*., 2000). Very 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 show in this manuscript that recombinant full-length FLAG tagged PrP from human, cattle or hamster from insect cells infected with recombinant baculoviruses appear predominantly dimeric under native conditions. Our recombinant PrP was purified under native conditions from the medium of insect cells infected with recombinant baculoviruses. 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.

PrP/PrP interactions in BHK cells transfected with recombinant SFV-RNAs

In order to prove the PrP/PrP interaction in highly developed eucaryotic 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 evolutionary 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 position 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) (Hundt *et al*., 2001).

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 carboxyterminal 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 1 J), we conclude that intermolecular disulfide bridges do not or only marginally contribute to PrP dimer formation.

Influence of additional octarepeats in PrP/PrP interaction processes

Additional octarepeats have been identified in patients suffering from familial CJD (Campbell *et al*., 1996; Goldfarb *et al*., 1993; Goldfarb *et al*., 1991; Owen *et al*., 1992; Owen *et al*., 1990). 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 behaviour. Expression of mutated PrP encompassing two, five and nine additional octarepeats in bait position versus wild-type PrP in prey position of the yeast two-hybrid system diminished slightly 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 inhibiton of the PrP/PrP interaction reflecting a to our knowledge hypothetical homozygous CJD patients 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 accumulates 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 additional nine octarepeats indeed have a pathogenic effect. Undimerized PrP^{c} may not be able to full-fill 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 carboxyterminal PrP/PrP interaction domain is then required for PrP^{Sc} to convert more free PrP^{c} molecules resulting in prion propagation. This requirement is full-filled 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, thus, that this binding domain might be important for 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 behaviour

Defined mutations within the human *Prn*-p gene such as the Leu102Pro mutation or the Asp178Asn mutation lead to GSS and FFI, respectively (for review see (Lasmézas and Weiss, 2000)). The polymorphism at position 129 Met/Val influences the susceptibility of humans towards FFI, fCJD and nvCJD. Interestingly enough, all patients suffering from nvCJD investigated so far are homozygous for methionine at this position. All these mutations and the polymorphism either expressed in both positions or in bait versus wild-type PrP in prey

position of the yeast two-hybrid system did not affect the PrP/PrP interaction process. This findings are in good harmony 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 carboxyterminal region stretching from aa90 to 230 in 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 therapy of TSEs. Also in 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*., 2000; Chiesa *et al*., 1998).

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 position of the yeast two-hybrid system. Bovine PrP interacted with human PrP, but hamster PrP failed to interact with bovine and human PrP (Table I). These data are in fairly good harmony with interspecies transmissions of prions. Indeed there is now convincing evidence from interspecies transmission studies in animals (Bruce *et al*., 1997; Lasmézas *et al*., 1996; Lasmezas *et al*., 2001) and transgenic mice (Hill *et al*., 1997; Scott *et al*., 1999) that cattle BSE prions have transmitted to humans (Table I). 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 I). 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 I). However, the ovine scrapie agent has been transmitted to transgenic mice expressing bovine PrP (Scott *et al*., 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 I). Although epidemiological data suggest that the ovine scrapie agent is hardly transmittable to humans, a sheep-human transmission which we suggest from our yeast two-hybrid data cannot be excluded. Employing the *in vitro* conversion system (Caughey *et al*., 1995) interconversion studies have been performed which 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 extend, into a proteinase K (pK) resistant form (Raymond *et al*., 2000) (Table I). Sheep PrPsen (AQ) was converted by PrP^{BSE} into the pK resistant state (Raymond *et al*., 1997) (Table I), whereas hamster PrPsen was not converted by bovine PrP^{BSE} (Raymond *et al*., 1997) (Table I). 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 interconversion 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 enlighten the role of PrP dimers in the replication mechanism of prions and the physiological function of PrP.

Table I: 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 transmissions in transgenic mice	Interspecies transmissions 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)

Materials and methods

Recombinant proteins generated in the Baculovirus system

FLAG::haPrP23-231 has been generated as described (Rieger *et al*., 1997). cDNAs encoding huPrP23-230, bovPrP25-242 obtained by H. Kretzschmar, Munich, 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 a *Bam*HI (5') and a *Pst*I (3') restriction site 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 for FLAG tagged haPrP previously (Rieger *et al*., 1997).

Recombinant pSFV plasmid constructions

Construction of SFV1-LRP::FLAG was described (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 codon 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 codon 223 - 225) at the 5' end, the tag-encoding sequence between codon 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 aminoterminal 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 have been 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, 10x 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 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 cotransfection 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 resuspended 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 transfered to a 0.4 cm cuvette. Performing cotransfection 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 BioRad Gene Pulser. The time constant after each pulse should be 0.4 to 0.5. The total volume of the electroporated cells ($8x10^6$) 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 14000 rpm 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 over night by rotating at 4°C, washed four times with PBS and eluted over night 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

Constructions 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::huPrP□GP lacking the octarepeat region aa 51-91 was generated via 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 a *Bam*HI (5') and a *Sal*I (3') restriction site. 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 length. This results in the insertion of 2 and 5 additional octarepeats. The constructs pSH2-1-GST::huPrP23-230P102L and pSH2-1-GST::huPrPM129V were cloned via 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-bovPrP via *Bam*HI (Krasemann *et al*., 1996) and *SalI*. 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 a 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 a β-galactosidase assays.

Size Exclusion chromatography (SEC)

Phast System (Amersham Pharmacia)–The Superose 12 PC 3.2/30 column (Amersham Pharmacia) was calibrated with the LMW calibration kit using the buffer 20mM 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 30µl/min and detected with a UV-M II monitor at 280nm. For denaturation FLAG::huPrP23-230 was incubated with 100mM DTT, 95°C for 15 minutes.

Antibodies

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.

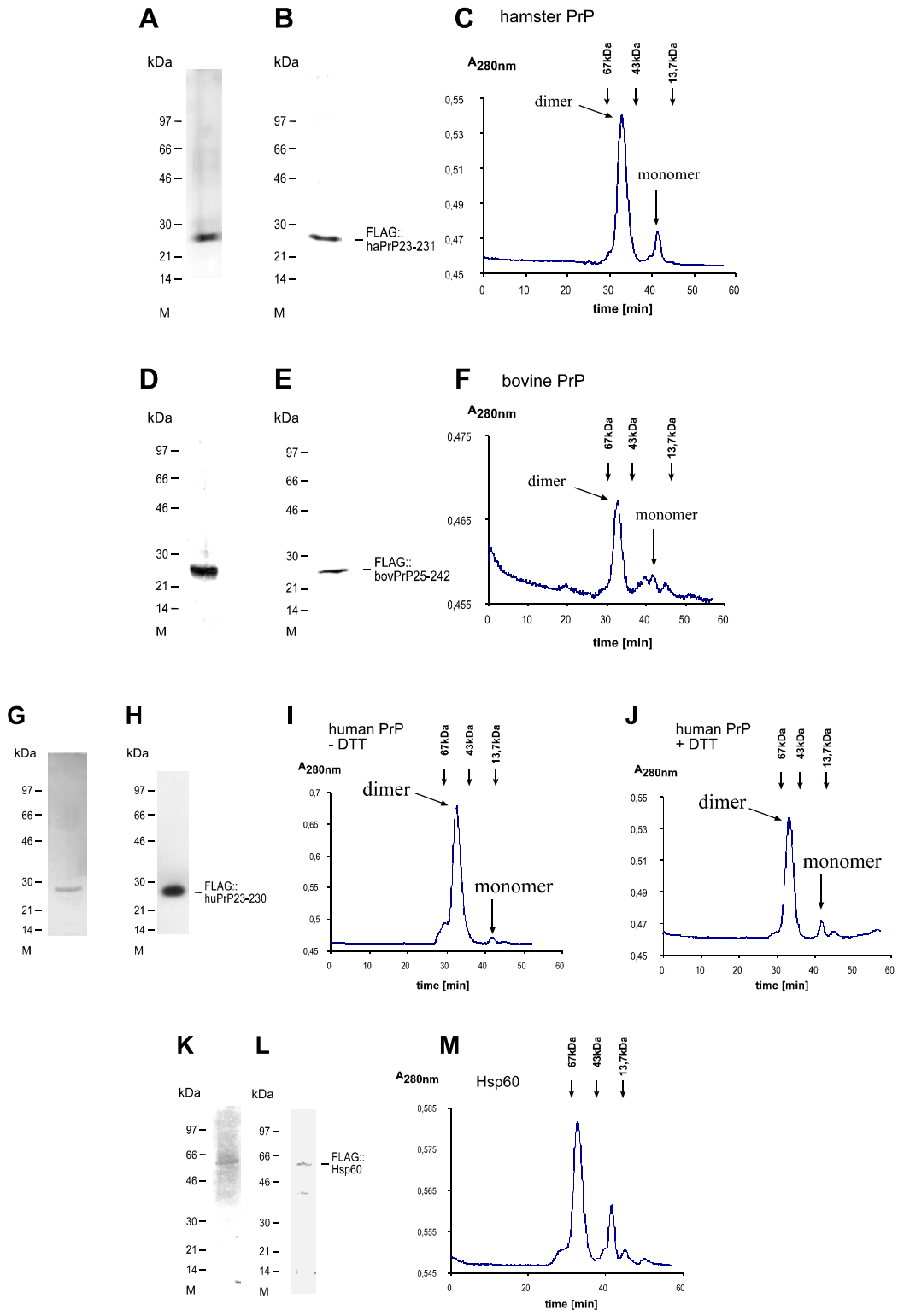

Fig. 1. Analysis of recombinant FLAG::PrP from hamster, cattle and man and FLAG::Hsp60 under native and denaturing conditions. 400 ng of purified FLAG::haPrP23-231 were analyzed by SDS-PAGE (12.5 % PA) followed by silver staining of the gel (**A**) and Western Blotting (**B**) employing the monoclonal 3B5 antibody. (C) 6 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. 600 ng of purified FLAG::bovPrP25-242 were analyzed by SDS-PAGE (12.5 %) followed by silver staining of the gel (D) and Western Blotting (E) employing the 3 B5 antibody. (F) 4 µg of non-denatured FLAG::bovPrP25-242 were analyzed by SEC as described above. 400 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) 8 µg of non-denatured FLAG::huPrP23-230 were analyzed by SEC in the absence of DTT as described above. (J) 6 µg of FLAG::huPrP23-230 were analyzed by SEC after denaturing with DTT. 300 ng of purified FLAG::HSP60 were analyzed by SDS-PAGE (12.5 %) followed by silver staining of the gel (K) and Western Blotting (L) employing an Hsp60 specific antibody. (M) 4 µg of non-denatured FLAG::Hsp60 were analyzed by SEC as described above.

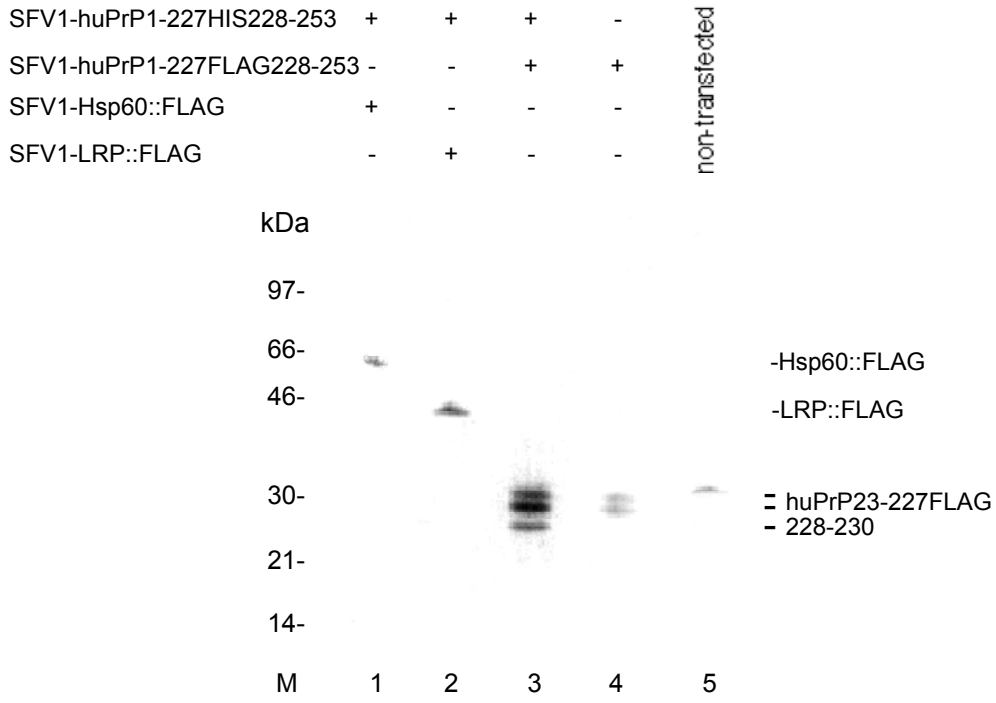

Fig. 2. Analysis of PrP/PrP interactions in BHK cells co-transfected with recombinant Semliki-Forest-Virus RNAs. Extracts of total protein from BHK cells either non-transfected (lane 5), transfected with SVF1-huPrP1-227FLAG228-253 (lane 4) or co-transfected with SVF1-huPrP1-227FLAG228-253 and SVF1-huPrP1-227HIS228-253 (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 a monoclonal anti-FLAG-antibody (M2).

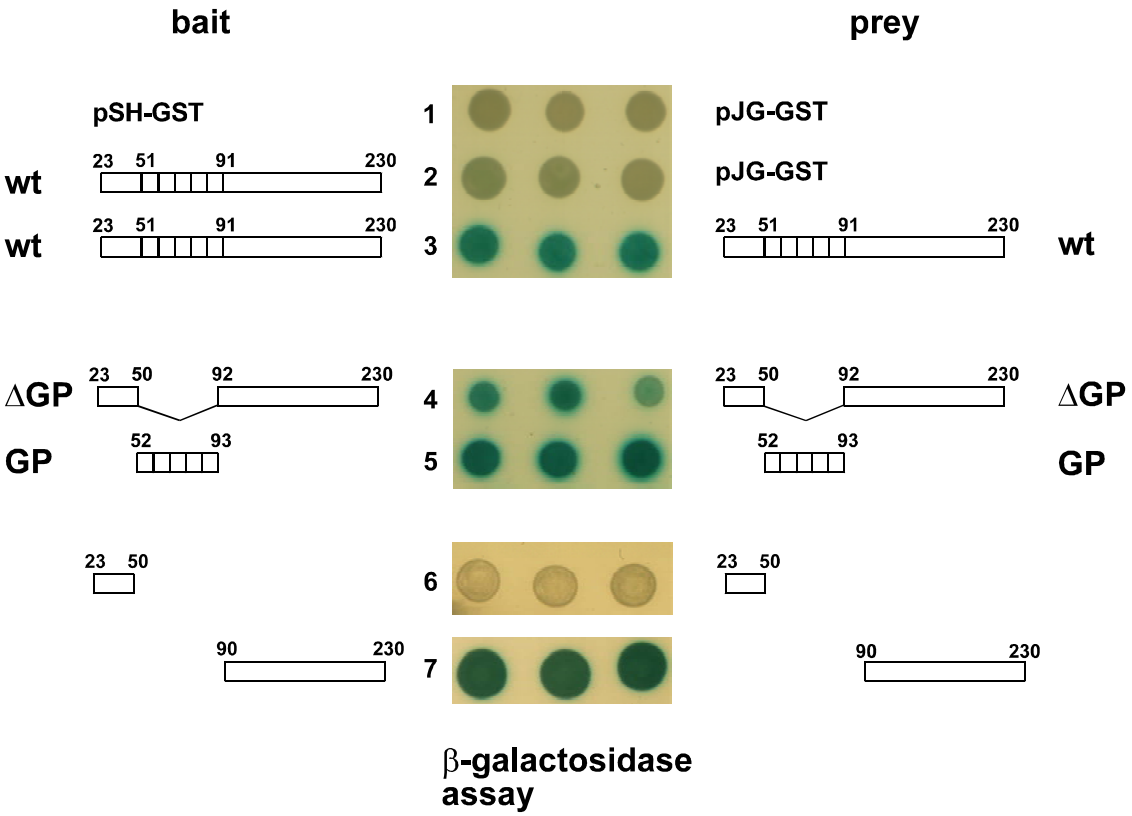

Fig. 3. Identification of PrP/PrP interaction domains in the yeast two-hybrid system (*S. cerevisiae*). Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (row 1 and 2), pJG-GST::PrP23-230 (row 3), pJG-GST::PrPΔGP (row 4), pJG-GST::GP52-93 (row 5), pJG-GST::PrP23-50 (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).

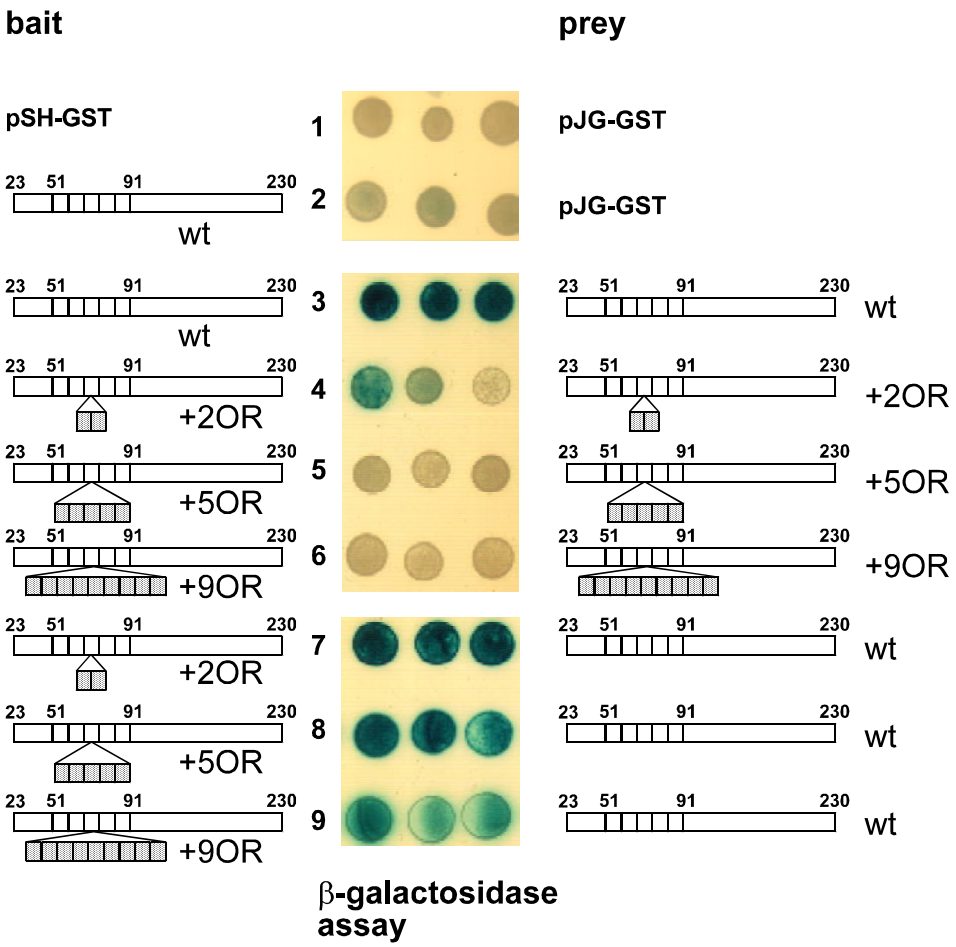

Fig. 4. Influence of additional octarepeats on the PrP/PrP interaction behaviour. Yeast cells encompassing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (rows 3, 7, 8 and 9), pJG-GST::PrP+2OR (row 4), pJG-GST::PrP+5OR (row 5), pJG-GST::PrP+9OR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

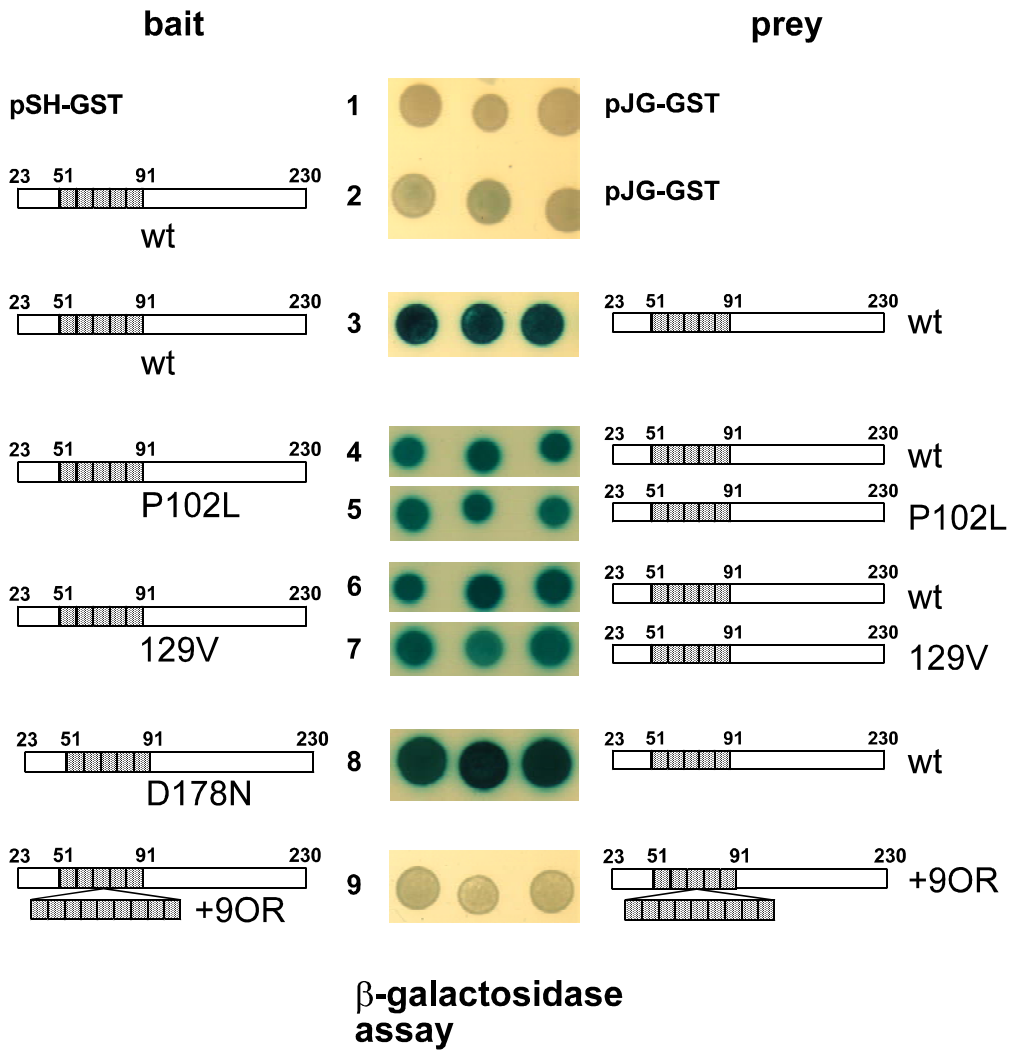

Fig. 5. Influence of the GSS-mutation P102L, the polymorphism M129V and the mutation D178N on the PrP/PrP interaction behaviour analyzed in the yeast two-hybrid system. Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (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).

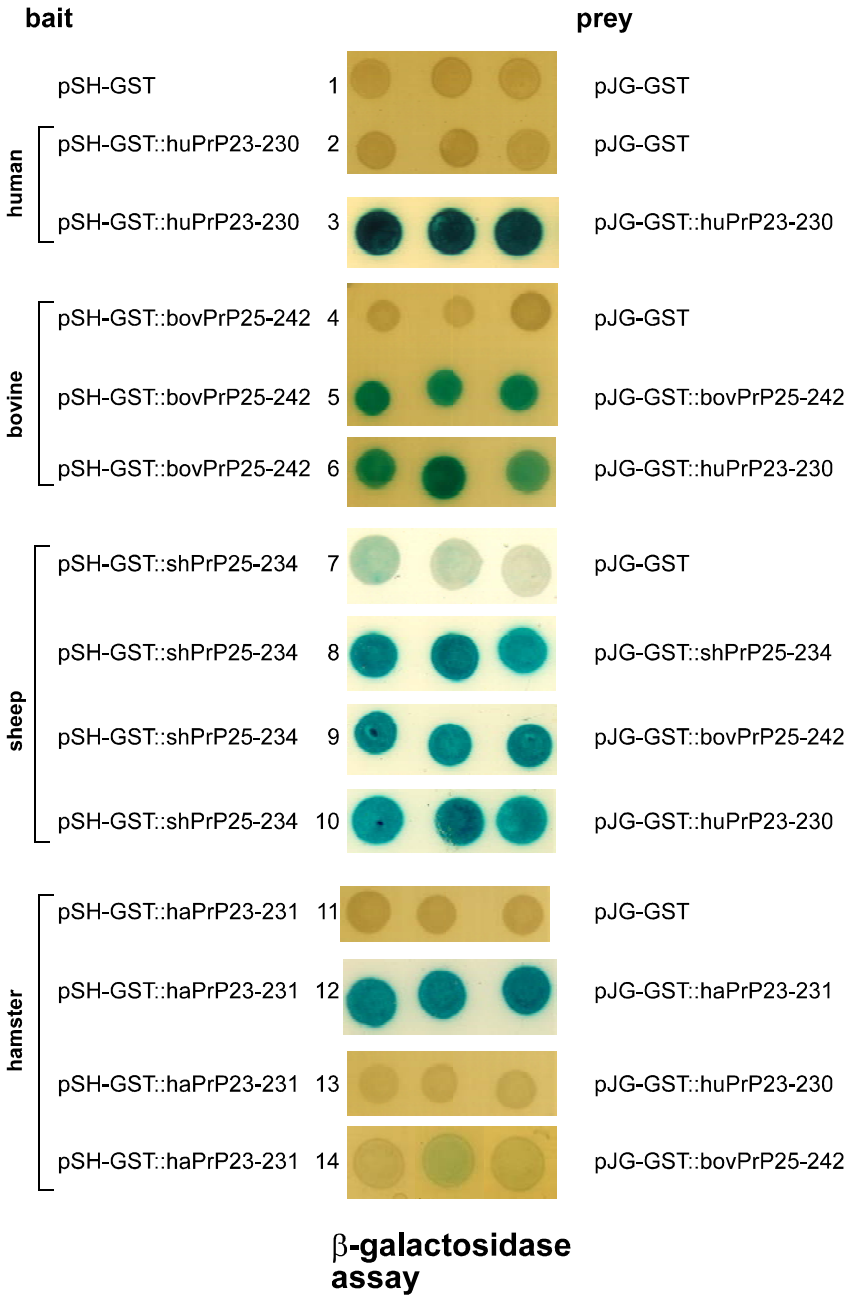

Fig. 6. Interaction analysis of PrP of different species in the yeast-two-hybrid system. The bait plasmids pSH-GST (row 1), pSH-GST::huPrP23-230 (rows 2 and 3), pSH-GST::bovPrP25-242 (rows 4-6), pSH-GST::shPrP25-234 (AQ) (rows 7-10) and pSH-GST::haPrP (rows 11-14) were co-transformed with the reporter-plasmid 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).

Chapter IV

High-level expression and characterisation of a glycosylated covalently linked dimer of the prion protein

submitted for publication:
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High-level expression and characterisation of a glycosylated covalently linked dimer of the prion protein. *Prot Eng* 2001

Abstract

There is evidence that prion protein dimers may be involved in the formation of the scrapie prion protein, PrP^{Sc}, from its normal (cellular) form, PrP^{c}. Very recently the crystal structure of the human prion protein in a dimeric form was reported (Knaus *et al.*, 2001). Here we report for the first time the overexpression of a human PrP dimer covalently linked by a FLAG peptide (PrP::FLAG::PrP) in the methylotropic yeast *Pichia pastoris*. FLAG-tagged human PrP (1-253) (huPrP::FLAG) was also expressed in the same system. Treatment with tunicamycin and endoglycosidase H showed that both fusion proteins are expressed as various glycoforms. Both PrP proteins were completely digested by proteinase K (pK), suggesting that the proteins do not have a PrP^{Sc} structure and are not infectious. Plasma membrane fractionation revealed that both proteins are exported to the plasma membrane of the cell. The glycosylated proteins could be a powerful tool for PrP^{c}/PrP^{Sc} conversion studies.

Introduction

Transmissible spongiform encephalopathies are fatal neurodegenerative disorders such as Creutzfeldt-Jacob disease in humans, bovine spongiform encephalopathy in cattle and scrapie in sheep or goat (Lasmézas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann and Aguzzi, 1997). They are associated with the accumulation of an abnormal form of the prion protein, PrP^{Sc}, derived from the normal cell surface glycoprotein PrP^{c} (Prusiner, 1982). PrP^{c} requires the 37kDa/67 kDa laminin receptor for internalization (Gauczynski *et al.*, 2001b), a process which is thought to require heperan sulfate proteoglycans (HSPGs) mediating the binding of PrP^{c} to its receptor via indirect binding domains (Hundt *et al.*, 2001). The conversion of PrP^{c} to PrP^{Sc} is thought to take place in compartments of the endocytic pathway such as endosomes, lysosomes or endolysosomes (for review see (Gauczynski *et al.*, 2001a)). PrP^{Sc} and PrP^{c} have very different biochemical properties. PrP^{c} is mainly α-helical and is readily degradable by proteinase K, whereas PrP^{Sc} is characterized by an increase in β-sheet conformation, a higher tendency to aggregate, insolubility and proteinase K resistance (Meyer *et al.*, 1986; Pan *et al.*, 1993; Prusiner *et al.*, 1984). In cases where the disease is transmitted, prion replication appears to involve the interaction between host PrP^{c} and pathogenic PrP^{Sc} from an external source (Prusiner *et al.*, 1984).

There is evidence that prion protein dimers may play a role in the conversion of PrP^{c} to PrP^{Sc}. Very recentlly the crystal structure of the human prion protein in a dimeric form was

reported (Knaus *et al.*, 2001). Formation of the dimer involves the three-dimensional swapping of helix 3 and rearrangement of the disulfide bond. The authors suggest that the 3D domain-swapping-dependent oligomerization may be an important step in the PrP^{c}/PrP^{Sc} conversion process. Formation of PrP dimers were also observed in N2a cells and in scrapie-infected hamster brains (Priola *et al.*, 1995). They have also been identified as intermediates in the PrP oligo-/multimerization process by fluorescence correlation spectroscopy (Post *et al.*, 1998) and molecular modelling suggested the existence of PrP dimers (Warwicker and Gane, 1996), which could be involved in interspecies transmission (Warwicker, 1997). Recently, covalently linked multimers were observed on Western blots of PrP^{Sc} purified from hamster brain infected with the 263K strain of scrapie (Callahan *et al.*, 2001). It was suggested that these multimers may be the result of some PrP molecules in the PrP^{Sc} aggregates becoming covalently crosslinked *in vivo.* Meyer *et al.*, observed a monomer-dimer equilibrium under native conditions in at least a fraction of PrP^{c} purified from bovine brains (Meyer *et al.*, 2000). Recently, a dimeric α-helical intermediate was observed during the in vitro conversion of recombinant hamster PrP to large insoluble aggregates (Jansen *et al.*, 2001).

The availablility of large amounts of recombinant PrP expressed in *E. coli* has allowed the solution structure of mouse, hamster, human and bovine PrP to be determined by NMR spectroscopy (Donne *et al.*, 1997; Lopez Garcia *et al.*, 2000; Riek *et al.*, 1997; Zahn *et al.*, 2000). However, these recombinant proteins lack two glycosyl groups and a glycosylphosphatidylinositol (GPI) membrane anchor. Very little is known about the effect of these two post-translational modifications on the structure and function of PrP.

In this study we expressed a covalently-linked human PrP dimer (PrP::FLAG::PrP) and full-length human PrP (huPrP::FLAG) in the methylotropic yeast, *Pichia pastoris*. This powerful expression system makes use of the highly inducible alcohol oxidase promoter to express large amounts of glycosylated protein. The proteins were expressed as fusion proteins to a FLAG peptide and the native prion signal sequence and GPI anchor were included to direct secretion of the protein. Expressions were carried out with tunicamycin, which blocks glycosylation in vivo, to confirm the mixed glycoform expression. Optimization of expression resulted in yields of approximately 50-100mg/l. The sensitivity of the expressed FLAG fusion proteins to proteinase K and endoglycosidase H was determined. The fusion proteins were detected in the yeast plasma membrane fraction but not in the media, suggesting secretion of the protein to the cell membrane.

Materials and methods

Reagents and antibodies

The monoclonal anti-PrP antibody 3B5 directed against the octapeptide repeat region of human and bovine PrP was a gift from G. Hunsmann, Göttingen, Germany, and the 3F4 antibody directed against aa 109-112 of hamster and human PrP was from Chemicon. Anti-FLAG antibody M2, secondary anti-mouse IgG-POD conjugate and tunicamycin were from Sigma. Proteinase K, endoglycosidase H and Pefablock were purchased from Roche Diagnostics.

Plasmid constructions

(1) Construction of pPICZB-huPrP1-227FLAG228-253. The insertion of a FLAG encoding sequence for the pSFV1-huPrP1-227FLAG228-253 plasmid is described elsewhere (Hundt *et al.*, in preparation). The cDNA was amplified by PCR from this plasmid, introducing *Eco*RI and *Xba*I restriction sites at the 5‘ and 3‘ ends. The amplified fragment was cloned into the *Pichia pastoris* expression plasmid pPICZB via *Eco*RI/*Xba*I restriction sites, resulting in pPICZB-huPrP1-227FLAG228-253.

(2) Construction of pPICZB-huPrP1-230FLAGhuPrP1-227FLAG228-253. cDNA encoding huPrP1-253 was amplified by PCR and cloned into pSFV1, as described (Krasemann *et al.*, 1996), resulting in pSFV1-huPrP1-253. The cDNA encoding huPrP1-230 was amplified by PCR from this plasmid, introducing *Eco*RI and *Hin*dIII restriction sites at the 5‘ and 3‘ ends. A second fragment (FLAGhuPrP23-227FLAG228-253) was amplied by PCR from the pSFV1-huPrP1-227FLAG228-253 plasmid, introducing a *Hin*dIII restriction site and a FLAG encoding sequence at the 5‘ end and an *Xba*I restriction site at the 3‘ end. These two fragments were restricted, ligated and cloned into the *Pichia pastoris* plasmid pPICZB via *Eco*RI/*Xba*I restriction sites, resulting in pPICZB-huPrP1-230FLAGhuPrP23-227FLAG228-253.

Expression in *Pichia pastoris*

The *P. pastoris* expression system uses the promoter from the alcohol oxidase gene, AOX1, to express heterologous proteins. The expression vector pPICZB (EasySelect Pichia Expression Kit, Invitrogen) was digested with *Eco*RI and *Xba*I and ligated to the inserts. DH5α cells were transformed with the ligation products and plated on low salt LB/zeocin medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, and 25 µg/ml zeocin). The

transformants were tested by restriction analysis, and positive clones were amplified to make larger amounts of DNA.

The nucleotide sequences of the resulting plasmids were confirmed by dideoxy sequencing. Prior to transformation into yeast, the plasmids were digested with *Sac*I. The DNA was transformed into *Pichia pastoris* (SMD 1168) according to the manufacturer's instructions and the cells were plated onto YPD/zeocin medium (1% yeast extract, 2% peptone, 2% D-glucose, 0.1mg/ml zeocin). For secondary selection of multicopy transformants using zeocin, clones were pooled, diluted in sterile water and about 1×10^4 cells were spread on YPD plates containing increasing concentrations (200, 400, 600 and 1000 µg/ml) of zeocin.

10 clones with high zeocin resistence were selected for a test expression. Single colonies were used to inoculate 10ml of BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base without amino acids, 0.00004% biotin, 1% glycerol, 50 µg/ml kanomycin, 0.1M potassium phosphate buffer, pH 6.0). The cultures were grown overnight at 28°C to an A_{600} of 2-6 and then harvested (2000g, 5 min, room temperature). The cultures were resuspended in medium that contained 0.5% methanol instead of glycerol in order to induce the yeast cells to express the heterologous protein. One ml aliquots of culture were removed every 24 hours and centrifuged at 6000 rpm for 2 minutes in a microcentrifuge. Sixty microlitres of the supernatant were added to 30µl of 3×SDS-loading buffer. The pellet was resuspended in 0.5ml distilled water and 60µl were added to 30µl 3×SDS-loading buffer. Expression of the recombinant protein was monitored by SDS-PAGE followed by Western blotting and detection with anti-PrP specific antibodies (3F4 or 3B5) or the anti-FLAG M2 antibody.

Larger-scale expression and optimization

The highest expressing clones of the covalently-linked dimer and monomer as determined by Western blot anysis were used to inoculate 25ml cultures of BMGY. The cultures were grown at 28°C (230rpm) to an A_{600} 2-6. After centrifugation the cultures were resuspended in 100ml BMMY containing 0.5%, 1.0% or 2% methanol (to an A600 of 1) in 1l baffled flasks and shaken at 28°C (200 rpm) for 72 hours. 1ml aliqouts were removed every 24 hours for analysis of protein expression.

Expression in the presence of tunicamycin

Tunicamycin was used to block *in vivo* glycosylation. It was added to 10ml cultures of the covalently-linked dimer and monomer (from a stock solution of 1mg/ml in 0.1M NaCl) to a final concentration of 15µg tunicamycin/ml culture. Small-scale expression was carried out

essentially as described above, with tunicamycin being included in the BMGY and BMMY culture media. 1ml aliquots were removed 24 hours after induction and expression of the covalently linked dimer and monomer in the cell lysate was analysed by SDS-PAGE and Western blotting. The monoclonal antibody 3B5, which recognises the octarepeat region of human and bovine PrP, was used for protein detection.

Cell lysis and sensitivity to proteinase K

Cell pellets containing over-expressed FLAG-tagged covalently linked dimer and monomer, isolated from 2ml of each culture were resuspended in 1ml lysis buffer (10 mM Tris/HCl buffer, pH 7.5, containing 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, and 0.5% deoxycholate). An equal volume of glass beads (500 microns) was added to each suspension and the cells were broken by vortexing for a total of 4 minutes in bursts of 30 s alternating with cooling on ice for 30 s. The glass beads were separated by centrifugation (4000 rpm for 10 mins, 4°C).

Resistance of the covalently linked PrP dimer and monomer to proteinase K was assessed. 100µl aliquots of the supernatants were incubated with proteinase K (0-4 µg/ml) at 37°C for 1 hour. Digestion was stopped by the addition of Pefablock to a final concentration of 1mM and samples were analysed by immunoblotting (with the 3B5 antibody) after of SDS-PAGE.

Sensitivity to endoglycosidase H

Cell pellets containing overexpressed FLAG-tagged covalently linked PrP dimer and monomer were lysed as above, but in the following lysis buffer; 40mM sodium citrate, pH 5.5, 0.05% SDS, 0.5 mM PMSF. 50µl aliquots of the supernatants were incubated with or without 0.5 units/ml endoglycosidase H at 37°C for 3 hours. The reaction was stopped by addition of 3 × SDS-loading buffer and heating to 95°C for 5 minutes. Deglycosylation was monitored by SDS-PAGE followed by Western blotting and detection with 3B5 antibody.

Purification of yeast plasma membrane fraction

The plasma membrane fractions of yeast overexpressing the FLAG fusion proteins were purified using standard procedures (Panaretou and Piper, 1996). *Pichia pastoris* culture pellets (from 50ml cultures) were resuspended in 10ml cold lysis buffer (25mM imidazole, pH 7.0, 2mM EDTA, 0.4M sucrose). The cells were re-pelleted by centrifugation and the supernatants discarded. Two ml of glass beads and 2ml of lysis buffer were added and cells were broken by vortexing as described above. 9ml of cold lysis buffer was added and the cell

debris and glass beads were pelleted by centrifugation (530g, 20 mins, 4°C). The supernatant was removed and centrifuged (22000g, 30minutes, 4°C) to pellet the plasma and mitochondria fractions. The supernant (cytosolic fraction) was removed and the pellet taken up in TBS containing 5% Triton X-100. This was further diluted to 20ml with TBS containing 0.1% sarcosine, 0.1% NP-40 and 100mM dithiothreitol.

Immunoprecipitation

The FLAG fusion proteins were immunoprecipitated with 200µl of a 50% slurry of protein A-Sepharose (Pharmacia) and 10µl of 3B5 antibody as described previously (Caughey *et al.*, 1999).

Removal of GPI anchor by cleavage with enterokinase

Enterokinase cleaves the final lysine of the FLAG-peptide and was used here to remove the GPI-anchor of huPrP::FLAG. The expressed dimer was also treated with enterokinase even though it has two potential cleavage sites. Yeast cells were lysed in TBS, 0.1% Triton X-100, and 100µl of each supernatant was incubated with $CaCl_2$ (final concentration 10mM) and enterokinase (50µl added, 1unit/µl) at 37°C for 20 hours. The reaction was terminated with EDTA (20mM).

SDS – polyacrylamide gel electrophoresis and immunoblotting

Protein samples were separated on 12% Mighty Small gels according to the manufacturer's protocol (Hoefer, Pharmaciea Biotech Inc. San Francisco, CA) and transferred electrophoretically onto pre-wetted polyvinyldifluoride membranes. The blots were incubated with an anti-PrP antibody (3F4, 3B5, 1:5000 dilution) or with an anti-FLAG M2 antibody (1:600 dilution). The incubation steps were preformed as described previously (Weiss *et al.*, 1995) and the bound antibody was visualized with 3,3'-diaminobenzidine tetrahydrochloride.

Results

Expression of covalently linked human PrP dimer and huPrP::FLAG proteins in Picha pastoris

A covalently linked dimer of the human PrP (PrP::FLAG::PrP), with the FLAG octapeptide (DYKDDDDK) as a linker and at its C-terminus (Figure 1A) was expressed in *Pichia pastoris*. The FLAG peptide is used as an epitope tag for detection and purification of recombinant proteins and was chosen here because of its highly charged, polar sequence. For comparison, we also expressed a C-terminally FLAG-tagged human PrP molecule (Figure 1B, huPrP::FLAG).

Plasmids pPICZB-huPrP1-227FLAG228-253 and pPICZB-huPrP1-230FLAGhuPrP1-227FLAG228-253, transformed into the protease deficient *P. pastoris* strain SMD 1168, exhibited high levels of intracellular production of the FLAG-tagged proteins (Figure 2). Antibody 3B5 (and also 3F4 and anti-FLAG M2, results not shown) recognized 3 bands with apparent molecular masses ranging from approximately 25 to 33kDa for huPrP::FLAG (Fig.2: lanes 1 and 2) and approximately 5 bands for PrP::FLAG::PrP (Fig.2: lanes 3 and 4), indicating that the fusion proteins were glycosylated. Higher molecular weight bands were also detected for huPrP::FLAG at approximately the same molecular weight as the dimer bands which suggests that the expressed PrP::FLAG forms covalently-linked dimers. Priola *et al.* (Priola *et al.*, 1995) also observed a 60-kDa PrP dimer derived from hamster PrP expressed in murine neuroblastoma cells. This 60-kDa PrP was not dissociated under several harsh denaturing conditions.

Optimum expression was obtained with a 0.5 - 1.0 % methanol concentration and an induction time of 24 hours (Figure 2). After longer induction times, degradation of the fusion proteins occurred. Our data represent the first high-level expression of PrP in *Pichia pastoris,* with an approximate expression yield of 50-100 mg fusion protein/l.

Effect of tunicamycin and endoglycosidase H sensitivity

HuPrP::FLAG has two potential glycosylation sites (N-X-S/T) whereas the covalently linked dimer has four sites. To investigate whether the higher molecular weight bands were due to glycosylated protein, we expressed the fusion proteins in media containing tunicamycin which blocks glycosylation *in vivo* and analysed the cell lysates by SDS-polyacrylamide gel electrophoresis and Western blotting (Figure 3). In the presence of tunicamycin there was no detectable glycosylated human PrP::FLAG (Fig. 3A). With the covalently-linked dimer, the bands corresponding to the tri- and tetraglycosylated forms were strongly reduced (Fig. 3B).

Endoglycosidase H cleaves high mannose sugars and was used to confirm the expression of various glycoforms of the fusion proteins. Cell lysate supernatants containing overexpressed huPrP::FLAG or PrP::FLAG::PrP were incubated with endoglycosidase H (0.5 units/ml) for 3 hours at 37°C. Separation of proteins by SDS-PAGE and immunodetection with the 3B5 antibody (Figure 4) showed no detectable higher molecular weight bands of huPrP::FLAG, corresponding to the mono- and diglycosylated forms. By contrast with the PrP::FLAG::PrP there is some residual glycosylation which may be consistent with the covalent prion dimer having some tertiary structure.

Proteinase K sensitivity

In order to analyse the resistance of the covalently linked dimer to proteinase K (pK) and to compare it with huPrP::FLAG expressed in the same system, the cell lysate supernatants were incubated with 0, 2 and 4 µg/ml pK for 1 hour at 37°C. Analysis by SDS-PAGE and Western blotting (Figure 5) showed that the fusion proteins have similar pK sensitivity, both being completely digested by 4µg/ml pK. Evidently pK is able to degrade the prion monomer and covalent dimer equivalently.

Secretion of the fusion proteins to the plasma membrane

The plasma membrane fractions of *Pichia pastoris* overexpressing huPrP::FLAG and PrP::FLAG::PrP were isolated and analysed by Western blotting (Figure 6B and C). Both fusion proteins were detected in the plasma membrane fractions. Coomassie blue staining confirms that the covalently linked dimer is overexpressed and exported to the cell membrane (Figure 6A, Lane 1).

Immunoprecipitation

The FLAG fusion proteins were immunoprecipitated with an anti-PrP antibody, 3B5, directed against the octapeptide repeat region of human and bovine PrP, and Protein A sepharose. The beads were washed and analysed by SDS-PAGE and immunoblotting (Figure 7), demonstrating that the various glycosylation forms of both the dimer and monomer are specifically recognised by PrP antibodies in solution, under non-denaturing conditions.

Enterokinase cleavage

Enterokinase is a highly specific serine protease which cleaves after the carboxy-terminal lysine of the recognition sequence Asp-Asp-Asp-Asp-Lys. This is the last five amino acids of

the FLAG –tag. Enterokinase was used to remove the final lysine of the FLAG peptide and the GPI anchor of huPrP::FLAG. The expressed dimer was also treated with enterokinase even though it has two potential cleavage sites.

Comparison of the digested HuPrP::FLAG (Figure 8, lane 2) with the undigested protein (Figure 8, lane 1) shows a slight reduction in molecular weight. However no difference in apparant molecular weights was observed in the case of the dimer (Figure 8, lanes 3 and 4). Since the dimer contains two FLAG-tags, one as the linker peptide and one close to the C-terminus, we would expect a reduction in the amount of dimer and the appearance of monomer bands after cleavage with enterokinase. The results obtained indicate that the dimer may have some tertiary structure, which might protect the internal cleavage site from the protease.

Discussion

In the present study we used the methylotropic yeast *Pichia pastoris* to express high-levels of non-, mono-, and diglycosylated full-length human PrP and various glycoforms of a covalently linked human PrP dimer. Over the last few years interest in the *P. pastoris* expression system has grown since it has the potential for high level expression. It has been reported that in some cases up to several grams of the target recombinant protein per litre of culture have been obtained (for review see (Romanos, 1995)), however it is normally necessary to carry out fermentation to achieve this level of protein expression.

In mammalian cells high mannose sugars are added to PrP^{c} in the endoplasmic reticulum and are subsequently modified in the Golgi, becoming endoglycosidase H resistant. In yeast, no modification of the high mannose sugars occurs and the glycosyl groups remain endoglycosidase H sensitive.

The physical state of the recombinant prion protein, monomer or covalent dimer, is unclear at present. The endoglycosidase H studies suggest that the covalently linked PrP dimer has some three-dimensional structure, stable enough to interfere with the deglycosylation by the enzyme. Equally the effects of tunicamycin in abolishing glycosylation are less complete with the covalent dimer. The proteinase K sensitivity status of FLAG tagged prion protein and the covalently linked PrP dimer, however, proved to be similar. This suggests neither recombinant protein has the PrP^{Sc} structure, which is pK resistant (Taraboulos *et al.*, 1990). The similarity in their cleavage properties is however not inconsistent with the covalent dimer retaining some tertiary structure.

The generation of a covalently linked enzymatically active dimer has been described for the protease of human immunodeficiency virus (HIV) type one, composed of two copies of the protease sequence linked by a structurally flexible hinge region (Krausslich, 1991). The expressed dimer was stable and active against HIV polyprotein substrates. It was reported recently that human PrP crystallizes in a dimeric form (Knaus *et al.*, 2001). Formation of the dimer involves 3D swapping of the C-terminal helix and rearrangement of the disulfide bond. The authors suggest that this oligomerization may be an important step in the PrP^{c}/PrP^{Sc} conversion process. We hypothesize that the covalently linked PrP dimer might be a useful tool in cell-free conversion assays (Horiuchi *et al.*, 2000). It could be used as a template in the assay or added to investigate whether the rate conversion of PrP^{c} to PrP^{Sc} is altered. In addition, a covalently linked PrP dimer might be a suitable tool in cell culture studies of non-infected or scrapie infected neuroblastoma cells, investigating again its role in the PrP^{c} and PrP^{Sc} propagation process.

Very recently, the 37/67 kDa laminin receptor has been identified as the cell surface receptor for cellular PrP (Gauczynski *et al.*, 2001b). This process might involve cell surface HSPGs identified as co-factors for PrP binding (Hundt *et al.*, 2001). The covalently linked PrP dimer might interfere with the PrP^{c}/PrP^{Sc} internalization process on neuronal cells.

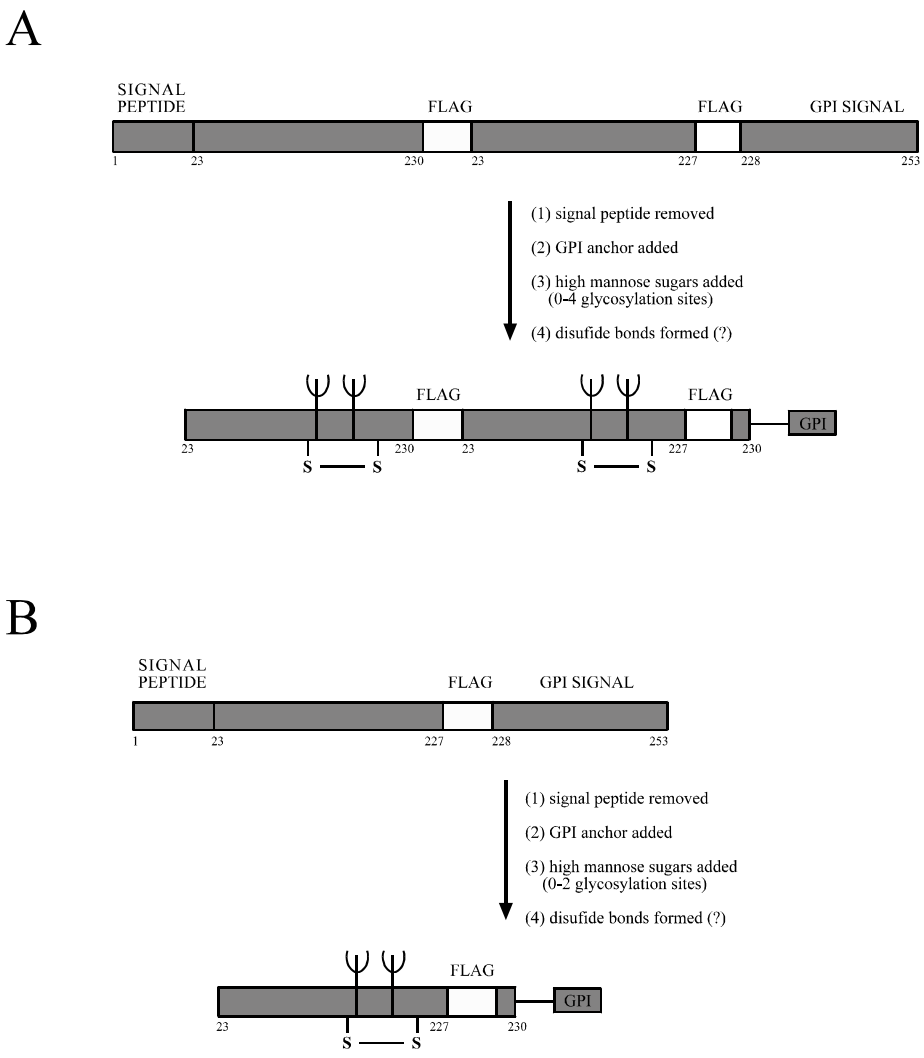

Fig. 1. Schematic diagram of FLAG-tagged PrP constructs and processing in the yeast cell. Both amino- and carboxyl terminal fragments are removed. The GPI anchor and high mannose glycans are added and the proteins are secreted to the cell surface. **(A)** Human PrP-covalently linked to another huPrP via a FLAG peptide linker. A second FLAG tag is located at the C-terminus, before the GPI anchor to aid detection and purification. The numbering of amino acid residues refers to the location on the untagged human PrP. **(B)** C-terminally FLAG-tagged human PrP.

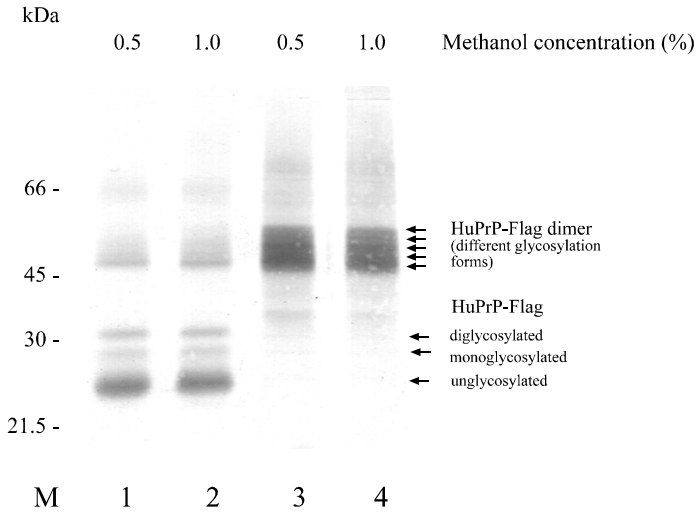

Fig. 2. Expression of FLAG-fusion proteins monitored by SDS-PAGE and Western blot analysis. Shown is a 12% polyacrylamide gel, immunodetection was carried out with the 3B5 antibody. (Lane 1) Lysate of cells expressing PrP::FLAG in 0.5% methanol; (Lane 2) PrP::FLAG in 1.0% methanol; (Lane 3) PrP::FLAG::PrP in 0.5% methanol and (Lane 4) PrP::FLAG::PrP in 1.0% methanol.

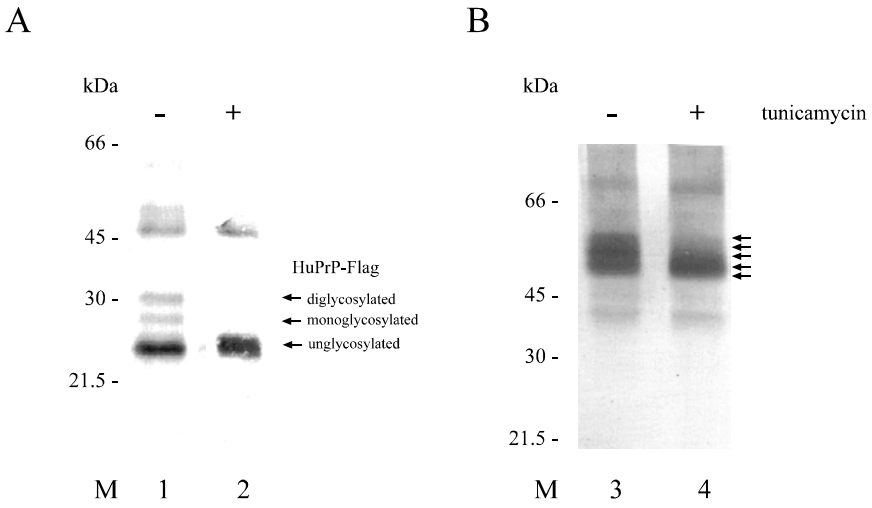

Fig. 3. Expression of FLAG-fusion proteins in the presence or absence of tunicamycin monitored by SDS-PAGE and Western blot analysis, immunodection was carried out with the 3B5 antibody. (Lane 1) Lysate of cells expressing PrP::FLAG in the absence and (Lane 2) in the presence of 15µg/ml tunicamycin. (Lane 3) Lysate of cells expressing PrP::FLAG::PrP in the absence and (Lane 4) in the presence of 15µg/ml tunicamycin.

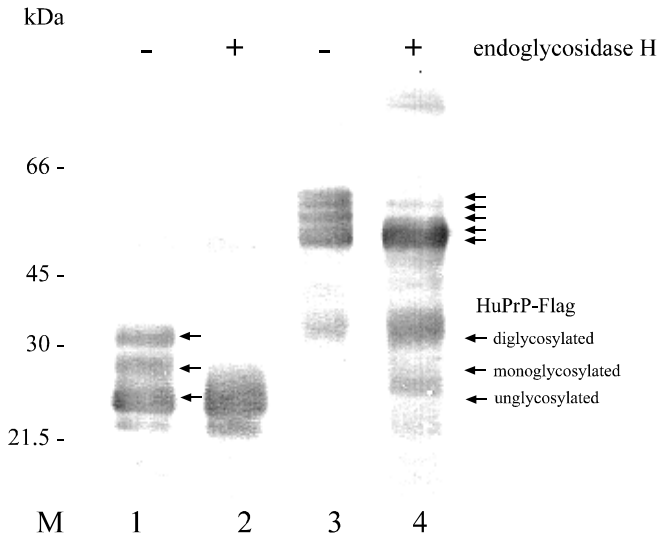

Fig. 4. Digestion with endoglycosidase H, monitored by SDS-PAGE and Western blot analysis. Immunodetection was performed with the 3B5 antibody. (Lanes1-2) Lysate supernatants of cells expressing PrP::FLAG, treated with 0 (Lane1) and 0.5 (Lane 2) units/ml endoglycosidase H. (Lanes 3-4) Lysate supernatants of cells expressing PrP::FLAG::PrP treated with 0 (Lane 3) and 0.5 (Lane 4) units/ml endoglycosidase H. At molecular weights less than 46 kDa a number of smaller bands are observed, these are most probably cleavage products. Note that the huPrP-FLAG monomer labels apply to lanes 1 and 2.

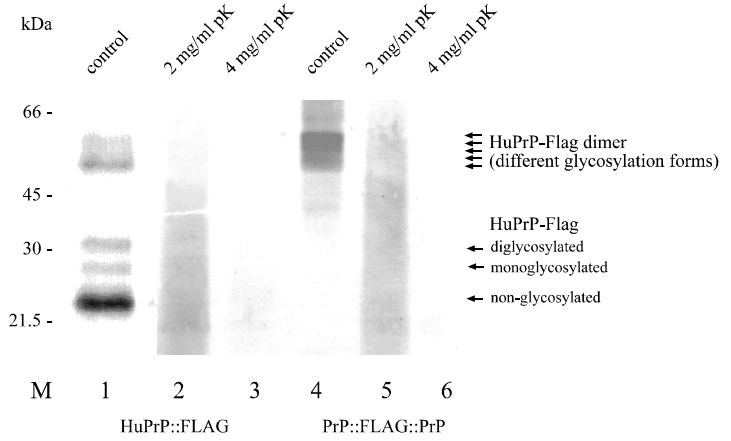

Fig. 5. Digestion with proteinase K, monitored by SDS-PAGE and Western blot analysis. Immunodection is with the 3B5 antibody. (Lanes1-3) Lysates of cells expressing PrP::FLAG, digested with 0 (Lane 1), 2 (Lane 2) and 4 (Lane 3) µg/ml proteinase K. (Lanes 4-6) Lysates of cells expressing PrP::FLAG::PrP digested with 0 (Lane 4), 2 (Lane 5) and 4 (Lane 6) µg/ml proteinase K.

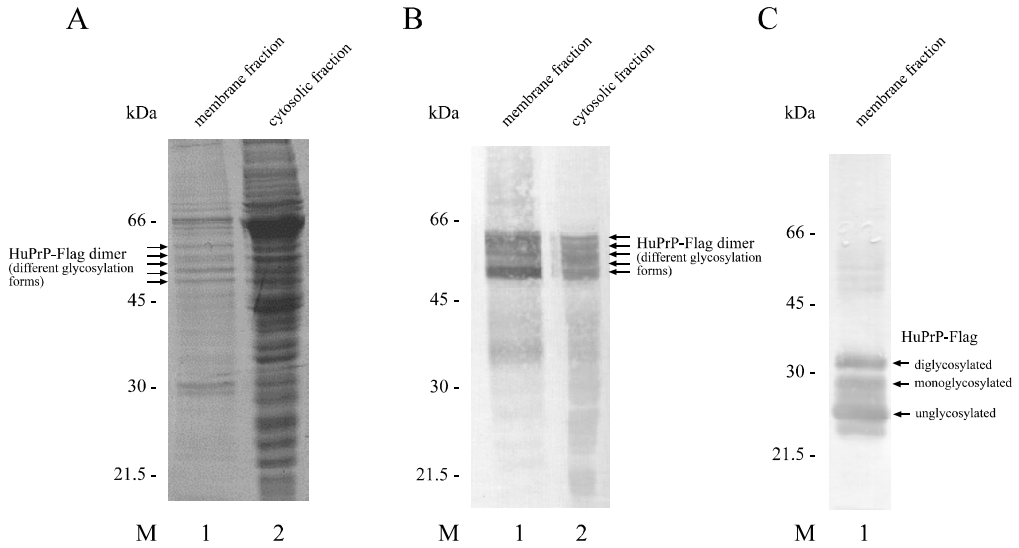

Fig. 6. Isolation of crude plasma membrane fractions **(A)** from yeast cells overexpressing PrP:FLAG::PrP, analysed by SDS-PAGE and Coomassie blue staining. (Lane 1) membrane fraction (Lane 2) cytosolic fraction, **(B)** analysed by Western blotting using the 3B5 antibody. (Lane 1) membrane fraction (Lane 2) cytosolic fraction. **(C)** Isolation of crude plasma membrane fraction from yeast cells overexpressing huPrP::FLAG analysed by Western blotting using the 3B5 antibody. (Lane 1) membrane fraction.

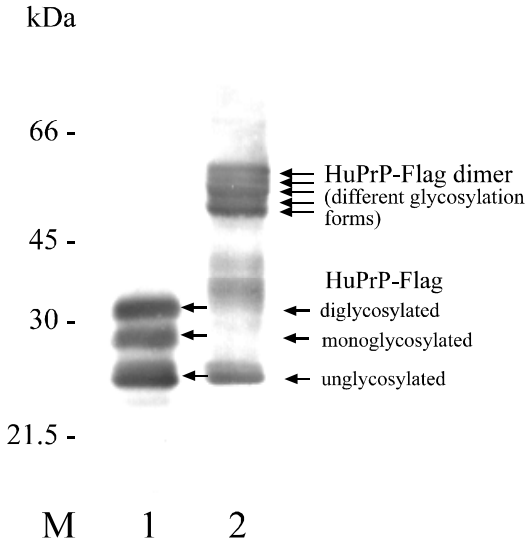

Fig.7. Immunoprecipitation of FLAG-fusion proteins monitored by Western blotting, with the 3B5 antibody. (Lane 1) huPrP::FLAG (Lane 2) PrP::FLAG::PrP.

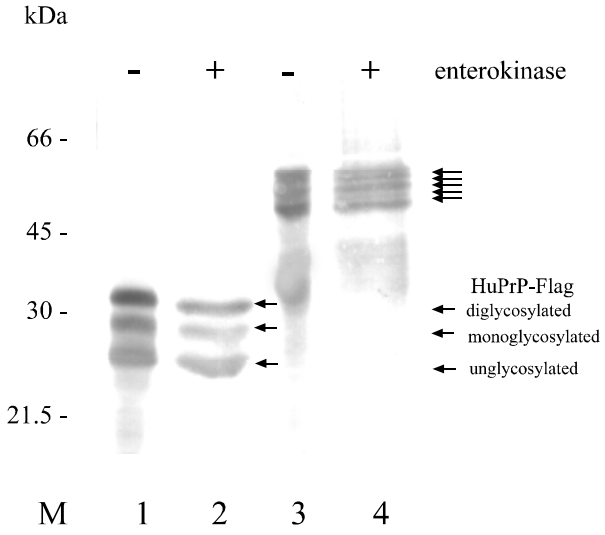

Fig. 8. Enterokinase cleavage of FLAG-fusion proteins. (Lane 1) untreated huPrP::FLAG, (Lane 2) enterokinase treated huPrP::FLAG, (Lane 3) untreated PrP::FLAG::PrP, (Lane 4) enterokinase treated PrP::FLAG::PrP.

Chapter V

Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor

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Abstract

Cell-binding and internalization studies on neuronal and non-neuronal cells have demonstrated that the 37-kDa/67-kDa laminin receptor (LRP/LR) acts as the receptor for the cellular prion protein (PrP). Here we identify direct and heparan sulfate proteoglycan (HSPG)-dependent interaction sites mediating the binding of the cellular PrP to its receptor, which we demonstrated *in vitro* on recombinant proteins. Mapping analyses in the yeast two-hybrid system and cell-binding assays identified PrP^{LRPbd1} [amino acids (aa) 144-179] as a direct and PrP^{LRPbd2} (aa53 to 93) as an indirect HSPG-dependent laminin receptor precursor (LRP)-binding site on PrP. The yeast two-hybrid system localized the direct PrP-binding domain on LRP between aa 161 and 179. Expression of an LRP mutant lacking the direct PrP-binding domain in wild-type and mutant HSPG-deficient Chinese hamster ovary cells by the Semliki Forest virus system demonstrates a second HSPG-dependent PrP-binding site on LRP. Considering the absence of LRP homodimerization and the direct and indirect LRP-PrP interaction sites, we propose a comprehensive model for the LRP-PrP-HSPG complex.

Introduction

We recently identified the 37-kDa laminin receptor precursor (LRP) as an interactor for the prion protein (PrP) (Rieger *et al*., 1997; for reviews see Rieger *et al*., 1999; Gauczynski *et al*., 2001a). Employing a series of neuronal and non-neuronal cells, we proved that the 37-kDa LRP/67-kDa high-affinity laminin receptor (LR) acts as the receptor for the cellular PrP (Gauczynski *et al*., 2001b). In the present manuscript we used the yeast two-hybrid system and cell-binding studies on neuronal as well as non-neuronal cells involving the Semliki-Forest-Virus (SFV) system (for review see Liljestrom and Garoff, 1991; Tubulekas *et al*., 1997) to identify domains on the PrP and the LRP involved in the PrP-LRP interaction on the cell surface. We identified two binding domains for LRP on PrP termed PrP^{LRPbd1} and PrP^{LRPbd2}. The first one binds directly to LRP, whereas the second one depends on the presence of heparan sulfate proteoglycans (HSPGs) on the cell surface. The yeast two-hybrid system and cell-binding assays on wild-type and mutant HSPG-deficient Chinese hamster ovary (CHO) cells also identified two binding domains for PrP on LRP .

The relationship between 37-kDa LRP and 67-kDa LR is not yet fully understood and has been explained with homodimerization of 37-kDa LRP (Landowski *et al*., 1995) or an additional factor, such as a polypeptide (Castronovo *et al*., 1991), which might bind to 37-kDa LRP to form the 67-kDa form of the receptor. The 67-kDa heterodimer might be stabilized by hydrophobic interactions mediated by fatty acids such as palmitate, oleate and stearate bound to 37-kDa LRP and to a galectin-3 (gal-3) cross reacting polypeptide (Buto *et al*., 1998; Landowski *et al*., 1995). However, we recently proved that the β-galactoside lectin gal-3 is not present on the surface of neuronal or non-neuronal cells used for PrP-binding/internalization studies (Gauczynski *et al*., 2001b) and anti-gal-3 antibodies failed to compete for the 37-kDa LRP/67-kDa LR-mediated binding and internalization of the cellular PrP (Gauczynski *et al*., 2001b) suggesting that gal-3 is not a partner of 37-kDa LRP in this context. In this study we investigated by a yeast two-hybrid system analysis whether gal-3 interacts with 37-kDa LRP and/or the cellular PrP. In addition, we investigated whether 37-kDa LRP interacts with itself in the yeast two-hybrid and analyzed the monomer/dimer status of the receptor by size-exclusion chromatography. Both PrP (Brimacombe *et al*., 1999; Caughey *et al*., 1994; Chen *et al*., 1995b; Gabizon *et al*., 1993) and the 37-kDa/67-kDa LR (Guo *et al*., 1992; Kazmin *et al*., 2000) bind to heparan sulfates. HSPGs are required for the binding of the fibroblast growth factor (FGF) to its FGFR receptor (Spivak *et al*., 1994; Venkataraman *et al*., 1999; Yayon *et al*., 1991) and act as initial attachment receptors for bacteria (Chen *et al*., 1995b) and viruses including alphaviruses (Byrnes and Griffin, 1998), human immuno-deficiency virus (HIV) type 1 (Mondor *et al*., 1998) and vaccinia virus (Chung *et al*., 1998). Heparan sulfates are components of amyloid plaques in prion diseases (Gabizon *et al*., 1993). We investigated the role of HSPGs as possible co-factors for 37-kDa LRP mediating PrP binding. We also constructed recombinant (rec.) SFV-vectors leading to the expression of an LRP mutant termed LRPdelBD::FLAG lacking the direct-binding domain for PrP in wild-type and mutant HSPG-deficient CHO cells. We compared the PrP-binding capacity to these cells with wild-type and mutant CHO cells hyperexpressing wild-type LRP::FLAG. In light of our findings that 37-kDa LRP fails to form homodimers, and that HSPGs mediate the binding of PrP to 37-kDa LRP, the relationship between 37-kDa LRP and 67-kDa LR might be explained by the association of LRP with HSPGs as outlined in a proposed model for the LRP-PrP-HSPG complex on the cell surface.

Results

Identification of a direct LRP interaction domain on PrP by the yeast two-hybrid system

To determine the domains of PrP interacting directly with LRP, we employed a yeast two-hybrid analysis with truncated PrP molecules in the bait position and LRP44-295 in the prey position. Only truncated PrP retaining the regions amino acids (aa) 144-179 (Figure 1A, rows 6 and 7) interacted with LRP. This region contains domains corresponding to the first α helix (aa 144-154), the second β-strand (aa 161-164) and the first amino acid of the second α helix (aa179-193) of the human prion protein (Zahn *et al*., 2000). Regions from aa 23 to 143 of the human PrP are not sufficient for binding to LRP (Figure 1A, rows 1-5). Regions from aa 180 to 230 of human PrP (row 8) are not required for the direct interaction between PrP and LRP. We termed this LRP interaction domain on PrP PrP^{LRPbd1}.

Retrenchment of the direct PrP-binding domain on LRP by the yeast two-hybrid system

Recently, we mapped a direct PrP-binding domain on LRP between aa 157 and 180 (Rieger *et al*., 1997) employing N-terminally truncated LRP molecules. In order to retrench this binding domain precisely we co-expressed the C-terminally truncated LRP molecules LRP44-101 and LRP44-160, respectively, together with full-length PrP in the yeast two-hybrid system. Both truncations failed to interact with PrP (Figure 1B) confirming that this direct PrP-binding site coincides with the laminin-binding domain (aa161-180). Expression of an LRP mutant lacking this direct PrP-binding domain (LRPdelBD161-180) in CHO cells (Figure 4K) showed that LRPdelBD161-180::FLAG was still able to bind to PrP, indicating the presence of a second binding site for PrP on LRP, which locates either between aa 101-160 or 181 and 295 of LRP.

PrP144-179 interacts directly with LRP161-179 in the yeast two-hybrid system

In order to prove a direct interaction between the PrP and the 37-kDa/67-kDa LRP/LR via PrP144-179 and LRP161-179, we co-expressed both protein domains in bait and prey position, respectively, resulting in a strong interaction (Figure. 1 C, row 1). In contrast, huPrP144-179 failed to interact with LRP180-295 (row 2) or LRP44-160 (row 3).

β-galactoside gal-3 does not interact with PrP or LRP in the yeast two-hybrid system

The association of gal-3 with the LRP has been suggested (Buto *et al*., 1998; Landowski *et al*., 1995). However, gal-3 antibodies do not influence the LRP-dependent binding/ internalization of PrP on the cell surface, suggesting that this molecule does not act as a co-receptor for LRP (Gauczynski *et al*., 2001b). For confirmation that gal-3 does not interact with PrP, we expressed gal-3 in bait and PrP in prey position of the yeast two-hybrid system resulting in no interaction between the two proteins (Figure 2A, row 2). Gal-3 also failed to interact with LRP in the yeast two-hybrid system (Figure 2A, row 3).

37-kDa LRP fails to interact with itself in the yeast two-hybrid system and appears monomeric by size-exclusion chromatography

The polymorphism of the LRP is still unclear. In order to test whether homodimerization of LRP could account for this 37-kDa/67-kDa polymorphism and to understand better the configuration of the LRP-PrP-binding complex, we cloned the cDNA encoding for LRP in the bait and prey position of the yeast two-hybrid system. LRP fails to interact with itself (Figure 2B, row 2), suggesting that LRP is unable to directly form homodimers. For confirmation we purified LRP::FLAG from SFV-RNA-LRP::FLAG transfected BHK cells by anti-FLAG antibody chromatography to homogeneity and analyzed the native protein by SDS-PAGE and size exclusion chromatography. The protein migrated as a 37-kDa protein on an SDS-polyacrylamide gel (Figure 2 C, lane 1) and eluted as a 40-kDa protein from a native size-exclusion column (Figure 2, D), confirming that 37-kDa LRP is monomeric under native conditions. Thus the 67-kDa form of the LRP may result from the association of the LRP with other molecules such as HSPGs.

Identification of the PrP interaction domains PrP^{LRPbd1} and PrP^{LRPbd2} by binding assays with prion peptides to NT2 and N2a cells

The yeast two-hybrid system identified the domain aa 144-179 of PrP as a direct binding site for LRP termed PrP^{LRPbd1}. To identify other domains of PrP, which might bind indirectly to LRP, we exposed NT2 and N2a cells to glutathione S-transferase (GST)-fused PrP peptides covering the entire PrP sequence. Besides peptide 129-175 encompassing the direct binding domain PrP^{LRPbd1}, GST::PrP53-93 bound to the cells in an LRP-dependent fashion. The binding of GST::PrP53-93 (Figure 3A) and GST::PrP129-175 (Figure 3C) is shown on NT2 cells in comparison with GST::PrP90-109 (Figure 3B). This binding can be inhibited by

addition of the LRP antibody W3 (insets in Figure 3A, C). The binding properties of the whole array of peptides are also shown on N2a cells (Figure 3D-J). Only GST::PrP53-93 (Figure 3E) and GST::huPrP129-175 (Figure 3H) bound to the cells dependent on LRP-LR (LRP antibody competition is shown in the bottom insets of Figure 3). The integrin laminin receptor VLA6 does not co-localize with PrP or LRP-LR on the surface of neuroblastoma cells (Gauczynski *et al*., 2001b). The addition of an anti-VLA6 antibody, failed also to compete for the binding of GST::PrP53-93 or GST::PrP129-175 to N2a cells (Figure 3 E and H, top insets), confirming that VLA6 does not act as a receptor for PrP. We termed the indirect binding domain PrP^{LRPbd2}. As the two binding domains are located N- and C-terminally of the proteinase K cleavage site of PrPres, we tested longer peptides in our binding assay corresponding to the two fragments that result from proteolytic cleavage of PrP, i.e. PrP23-89 and PrP90-230. Both peptides bound to both cell types in an LRP-dependent manner (Table I). Combining these data with the results from the yeast two-hybrid system (Figure 1) we conclude that two binding sites on PrP for LRP termed PrP^{LRPbd1} (aa144 to 179) and PrP^{LRPbd2} (aa53-93) do exist. Results of the PrP peptide binding studies to N2a and NT2 cells including antibody competitions are summarized in Table I.

Table I: Summary of the binding behaviour of individual GST-fused PrP peptides to NT2 and N2a cells including LRP-LR and VLA6 antibody competition

Peptide (aa)	Binding to N2a cells	LRP-LR antibody competition	Binding to NT2 cells	LRP-LR antibody competition
PrP53 -93	+++	+++*	+++	+++
PrP90-109	-	-	-	-
PrP110-128	-	-	-	-
PrP129-175	+++	+++*	+++	+++
PrP180-210	-	-	-	-
PrP218-230	-	-	-	-
PrP23-89	+++	+++	+++	+++
PrP90-230	+++	+++	+++	+++

*No competition with an VLA6 antibody
+++, strong binding/competition
-, no binding/competition

Binding of PrP to LRP via PrP^{LRPbd2} is dependent on HSPGs

The cell-binding assay led to the identification of an additional binding domain for LRP on PrP which was not identified in the yeast two-hybrid system, indicating that a third molecule is necessary to mediate the binding of LRP to PrP^{LRPbd2}. It has been reported that 60% of the binding of rec. chicken PrP to CHO cells depends on the presence of endogenous heparan sulfates (Shyng *et al*., 1994). Mutant CHO cells (S745) are severely deficient in HSPGs because of an altered xylose transferase activity, the first enzyme required for glycosylaminoglycan (GAG) synthesis (Esko *et al*., 1985) and therefore represent an appropriate model system to investigate whether HSPGs might represent the third interactor in the binding of PrP^{LRPbd2} to LRP. First, we proved that the binding of rec. GST::huPrP23-230 to wild-type CHO cells (Figure 4A) and to the mutant CHO cells (Figure 4B) was LRP-LR-dependent (Figure 4C and D). We then saturated selectively PrP^{LRPbd1} by incubating GST::huPrP23-230 with a monoclonal PrP antibody directed against the domain 140-180 of PrP. Obstructing PrP^{LRPbd1} inhibited the binding of the rec. PrP to HSPG-deficient (Figure 4F) but not to wild-type CHO cells (Figure 4E), demonstrating that binding of PrP to LRP via PrP^{LRPbd2} needs the presence of HSPGs. The peptide GST::PrP53-93 corresponding to PrP^{LRPbd2}, which bound to normal cells (Figure 4G), did not bind to HSPG-deficient cells (Figure 4H). However, the binding was restored in a dose-dependent manner after addition of soluble HSPGs (Figure 4I and J), confirming that the interaction of PrP to LRP via PrP^{LRPbd2} is HSPG-dependent.

Identification an HSPG-dependent second binding site for PrP on LRP

The two binding sites on PrP, one of which is direct and the other indirect, suggested that two ‚acceptor' sites might also exist on LRP. To test this hypothesis we adapted our SFV expression system to CHO cells. We then expressed LRP::FLAG and a mutant LRP lacking the direct PrP-binding domain (aa 161-180), termed LRPdelBD::FLAG, in wild-type CHO cells and the mutant CHO-S745 cell line lacking HSPGs (Figure 4K, lower panels). In wild-type CHO cells (Figure 4K, lanes 1-6) the binding of GST::huPrP was enhanced, when LRP::FLAG was hyperexpressed (Figure 4K, lane 2 versus lane 6). Hyperexpression of LRPdelBD::FLAG did not reduce the amount of the bound GST::huPrP (Figure 4K, lane 4), indicating that the binding was HSPG mediated. In mutant CHO-S745 cells lacking HSPGs (Figure 4K, lanes 7-13) the amount of bound GST::huPrP was also enhanced in LRP::FLAG hyperexpressing cells compared with non-transfected cells (Figure 4K, lane 8 and 13). CHO-

S-745 cells expressing LRPdelBD::FLAG, however, showed a reduced binding of GST::huPrP (Figure 4K, lane 10), similar to non-transfected cells (Figure 4K, lane 13) suggesting that the second indirect PrP-binding site was not functioning in the absence of HSPGs. In order to confirm that HSPGs are responsible for the binding of PrP to the second binding domain on LRP, we added HSPGs to cells overexpressing LRPdelBD::FLAG resulting in a total restoration of the PrP-binding (Figure 4K, lane 11). HSPGs failed to increase the binding of GST::huPrP to CHO wild-type (Figure 4L, lanes 2 and 3) and CHO-S-745 cells (Figure 4L, lanes 5 and 6) due to the presence of the direct binding domains on PrP and LRP. We conclude from these data that two binding sites for PrP on LRP exist: a direct one, which is located from aa161-179 and a second indirect one which resides either between aa101-160 or between aa 180-295 of LRP.

Interaction of PrP and LRP *in vitro*

The direct binding domains on LRP (aa161-179) and PrP (aa144-179) should allow the two proteins to interact with each other *in vitro*. GST-fused LRP (Rieger *et al*., 1997) and immobilized FLAG::huPrP (Figure 4 M, lanes 1 and 2) were able to interact with each other *in vitro* as shown in the pull down assay depicted in Figure 4 M. As already observed on wild-type CHO and CHO-S-745 cells, HSPGs did not influence the interaction due to the presence of the direct interaction domains (Figure 4M, lanes 2 and 3). However, HSPGs did affect the LRP-PrP53-93 interaction (HSPG-dependent binding domain on PrP) and the LRPdelBD-PrP interaction (lacking the direct binding domain on LRP) in CHO-S-745 cells (Figure 4G-J and K, respectively).

Discussion

Cell-binding and internalization studies proved that the 37-kDa LRP/67-kDa LR acts as the receptor for the cellular PrP^c, on the cell surface (Gauczynski *et al*., 2001b). In order to investigate the interaction domains on PrP and LRP-LR mediating the binding of these two proteins a series of interaction studies employing the yeast two-hybrid system as well as PrP-binding assays with neuronal and non-neuronal cells including the SFV-system have been performed.

Mapping of the LRP interaction sites on PrP

First, we aimed to determine which part of the PrP interacts with LRP. We performed a yeast two-hybrid analysis with a series of PrP deletion variants and employed a series of PrP peptides covering the entire PrP in various cell-binding assays.

In the yeast two-hybrid system, we identified the PrP domain aa144-179 as a direct LRP-binding domain, termed PrP^{LRPbd1}. This binding domain was confirmed in cell-binding assays in N2a and NT2 cells with PrP peptides encompassing the entire PrP sequence. As already observed with full-length PrP (Gauczynski *et al*., 2001b), the staining pattern with the PrP peptides to N2a and NT2 cells is also punctuate due to either receptor clustering or PrP-PrP peptide aggregation, or both. Receptor clustering was observed with a variety of other cell-surface receptors such as the FGF receptor (Utton *et al*., 2001), the muscle nicotinic acetylcholine receptor (AChR) (Hoch *et al*., 2001) or the tumor necrosis factor receptor (TNFR55) (De Wilde *et al*., 2001). In addition to the yeast two-hybrid assay, which identified the direct PrP-LRP-LR interaction domain PrP^{LRPbd1}, the cell-binding assay identified a second binding domain between aa53 and aa93 of PrP, termed PrP^{LRPbd2}. This domain was not functional in the yeast two-hybrid system, indicating that an additional factor lacking in the yeast cell nucleus is required for mediating the interaction between LRP and PrP through PrP^{LRPbd2}. Employing a mutant HSPG-deficient CHO cell line, in the binding assay revealed that PrP^{LRPbd2} binds to LRP via HSPGs. This finding was confirmed by using the PrP peptide 53-93 corresponding to PrP^{LRPbd2}. PrP53-93 failed to bind to the mutant CHO cells but the binding was restored in a dose-dependent manner by the addition of soluble HSPGs.

Our mapping data, which resulted in the identification of two LRP-binding sites on PrP, are consistent with the results obtained with PrP knock-out mice expressing PrPs with N-proximal deletions, suggesting that PrP could bind to its natural ligand, termed Lprp which could represent a PrP receptor, in a region C-terminal to aa 134 of the PrP (Shmerling *et al*., 1998). In line with this study, PrP^{LRPbd1} would correspond to the Lprp-binding domain. The same authors hypothesized the existence of a second domain located more N-terminally initiating a signal transduction necessary to maintain normal cellular functions. Recently, a signal transduction activity of the PrP by activation of the tyrosine kinase Fyn was described (Mouillet-Richard *et al*., 2000). As PrP resides as a GPI-anchored protein outside the cell, whereas the tyrosine kinase Fyn locates to the inner plasma membrane inside the cell, transmembrane orientated LRP-LR might be a reasonable candidate mediating the intracellular signal transduction between PrP^{c} and Fyn. The cytosolic domain of N-syndecans

(syndecan-3), one of a family of four transmembrane cell surface HSPGs, has recently been shown to bind to complexes containing c-Src and Fyn kinases (Kinnunen *et al*., 1998). This activity is related to neurite outgrowth (Kinnunen *et al*., 1998). Hence, it is also conceivable that HSPGs might be necessary for the so far hypothetical LRP-LR-mediated signal transduction between PrP^c and Fyn. A possible intercellular role of the PrP-LRP-LR interaction involving HSPGs which might result in signalling or cell attachment (for review see (Gauczynski *et al*., 2001a) will be further investigated in detailed cell-cell interaction assays.

Recently, studies on the transmission of human prions to transgenic mice suggested that a so far unidentified protein X may participate in the formation of the PrPres. Substitution of residues 167, 171 and 218 prevented PrPres formation suggesting that protein X may interact with those residues of the prion protein (Kaneko *et al*., 1997b). As aa 167 and 171 reside within the direct PrP-LRP interaction domain termed PrP^{LRPbd1} it cannot be excluded that LRP-LR might function as protein X. Application of LRP-LR in PrP oligo/multimerization processes may enlighten a possible role of the 37-kDa/67-kDa laminin receptor in PrPres formation.

Mapping of the PrP interaction sites on LRP

A yeast two-hybrid analysis with C-terminal LRP truncations extended a previous analysis (Rieger *et al*., 1997) and retrenched that the direct PrP-binding domain to aa161-179 of LRP. As the binding domain PrP^{LRPbd2} on PrP is HSPG-dependent, we hypothesized that a second indirect binding domain for PrP may also exist on LRP. To test this hypothesis, we analyzed PrP-binding to wild-type CHO cells, to mutant HSPG-deficient CHO cells and to both cell types hyperexpressing either the full length LRP::FLAG or an LRP mutant lacking the direct PrP-binding domain (aa161-180), termed LRPdelBD::FLAG. HSPGs failed to increase the binding of GST::huPrP to CHO wild-type and CHO-S745 cells due to the presence of the direct binding domains on PrP and LRP. CHO and CHO-S745 cells transfected with recombinant SFV RNAs further revealed the role of HSPGs in the binding of PrP to LRP. Hyperexpression of LRP::FLAG or LRPdelBD::FLAG in wild-type CHO cells resulted in approximately the same increase of GST::huPrP-binding when compared with non-transfected cells. This indicates that binding of PrP to LRP::FLAG can occur via a second PrP-interaction domain present in LRPdelBD::FLAG. In HSPG-deficient CHO cells, however, PrP-binding to cells hyperexpressing LRPdelBD::FLAG was similar to non-

transfected cells, indicating that the second interaction domain on LRP was not functioning. In contrast, LRP::FLAG hyperexpressing HSPG-deficient CHO cells showed an increased binding of GST::huPrP due to the presence of the direct HSPG-independent PrP-binding domain (aa161-179). The addition of HSPGs to LRPdelBD::FLAG hyperexpressing HSPG-deficient CHO cells restored GST::huPrP-binding to levels achieved with LRP::FLAG hyperexpressing cells. The data demonstrate the existence of a second HSPG-dependent PrP-binding domain on LRP, residing either between aa101 and 160 or aa181 and 285 of LRP. The identification of a HSPG-binding domain on LRP-LR which resides between aa 205 and 229 of LRP-LR (Kazmin *et al*., 2000) suggests that this domain may represent the HSPG-dependent-binding site for PrP. A monoclonal antibody directed against aa 167-243 of LRP reduces PrP-binding to neuronal cells (Gauczynski *et al*., 2001b) suggesting that the indirect binding domain may reside between aa180 and 285 rather than aa101 to 160 of LRP.

Interaction of PrP and LRP *in vitro*

Rec. purified PrP and LRP interact with each other *in vitro* due to the presence of the direct PrP-LRP interaction domains. The influence of HSPGs on the indirect PrP-LRP interaction domains, however, was only detectable on the HSPG-deficient CHO-S745 cells employing PrP53-93 (representing the indirect binding domain on PrP) and LRPdelBD (lacking the direct binding domain on LRP).

The relationship between 37-kDa LRP and 67-kDa LR

Attempts to isolate the gene for the 67-kDa LR revealed a cDNA fragment encoding the 37-kDa LRP (Grosso *et al*., 1991; Rao *et al*., 1983; Yow *et al*., 1988). Pulse-Chase experiments with 37kDa LRP specific antibodies demonstrated that 37-kDa LRP is the precursor of 67-kDa LR (Castronovo *et al*., 1991; Rao *et al*., 1989). The 37-kDa molecule encoded by the full-lengh gene identified for the LR is virtually identical to the ribosomal protein p40. The 37LRP/p40 evolved from a ribosomal protein essential for protein synthesis lacking any laminin-binding abilities, to a laminin-binding cell surface receptor (for review see (Ardini *et al*., 1998). The molecular structure of the 37-kDa LRP/67-kDa LR and the mechanism by which the 37-kDa LRP forms the mature 67-kDa LR remains unclear. Our data from a yeast two-hybrid analysis show that LRP fails to interact with itself, an argument against the hypothesis of a direct homodimerization. In addition, homogeneous rec. LRP::FLAG appears to be monomeric as analyzed by size-exclusion chromatography. Although we only used 0.1

% Triton-X-100 in the purification procedure, we cannot exclude that this small amount of non-ionic detergent may disrupt a native dimeric state of the receptor. A recent study suggested that acylation of LRP would allow it to associate with a heterologous molecule (Buto *et al*., 1998). In the light of our results, the relationship between the 37-kDa LRP and 67-kDa LR might be explained by the association of an LRP molecule with heparan sulfates.

Role of heparan sulfates in the PrP-LRP interaction process

The binding of chicken PrP to the surface of mammalian cells has been shown to depend partly on the availability of heparan sulfates expressed by these cells (Shyng *et al*., 1995). PrP interacts with heparan sulfates (Brimacombe *et al*., 1999; Caughey *et al*., 1994; Chen *et al*., 1995a; Gabizon *et al*., 1993). Recent studies demonstrated that the binding of copper to PrP which occurs within the octarepeat region (Brown *et al*., 1997) (corresponding to PrP^{LRPbd2}) can be competed by the addition of HSPGs (Brimacombe *et al*., 1999), confirming that this region of PrP binds to HSPGs. Finally, LRP-LR has also been shown to be a heparin/heparan sulfate-binding molecule (Guo *et al*., 1992; Kazmin *et al*., 2000). The predicted α-helical structure (aa205-aa229) of LRP-LR is proposed to have heparin binding characteristics (Kazmin *et al*., 2000). The requirement of heparin-like molecules for the formation of a ligand-receptor complex is not unprecedented and is well illustrated by the example of the binding of the FGF to its receptor, FGFR (Yayon *et al*., 1991; Spivak *et al*., 1994; Venkataraman *et al*., 1999). All these data match very well with our findings, providing a comprehensive model of the PrP-LRP-LR interaction (Figure 5). A heparan sulfate arm of a cell-surface HSPG molecule might be located between PrP^{LRPbd2} and the indirect binding domain of LRP to create a sandwich interaction site, whereas PrP would interact with LRP-LR (aa161-179) directly via PrP^{LRPbd1}. We cannot exclude that HSPGs intervene in a more indirect manner, by changing the conformation of LRP-LR as to render it amenable to its interaction with the PrP^{LRPbd2} region of PrP.

Components blocking the direct and indirect PrP-LRP interaction domains on PrP and LRP may represent a novel class of molecules suitable for a therapeutic intervention in prion diseases.

Materials and methods

Construction of pSFV1-LRPdelBD::FLAG- preparation of SFV mRNAs *in vitro*

The LRP mutant pSFV1-LRPdelBD::FLAG (deletion of the direct PrP-binding domain located between aa161 and aa180) was generated by the QuikChange™ site-directed mutagenesis method (Stratagene) using the pSFV1-LRP::FLAG plasmid DNA (Gauczynski *et al*., 2001b) as template for PCR and confirmed by dideoxy sequencing. The rec. plasmid DNAs pSFV3-lacZ (Life Technologies), pSFV1-LRP::FLAG and pSFV1-LRPdelBD::FLAG were linearized, purified and transcribed as described (Gauczynski *et al*., 2001b). The correct length of the transcripts was verified by agarose gel electrophoresis. RNA was stored at -20°C.

Mammalian cell culture, transfection and co-transfection studies with the SFV system

Mutant CHO cells (S745) deficient in xylose transferase (Esko *et al*., 1985) as well as wild-type CHO (K1) were cultivated in NUT.MIX.F-12(HAM) supplemented with GLUTAMAX-I (GIBCO-BRL), 10 % fetal calf serum (FCS), 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5 % CO_2. Transfection and co-transfection were carried out as described (Gauczynski *et al*., 2001b). Transfection efficiencies as determined by transfecting SFV3-*lac*Z control RNA followed by X-gal staining were ~ 80% for CHO-K1 or CHO-S745 cells.

Purification of LRP::FLAG from the SFV system

Transfection of BHK cells with the rec. SFV LRP::FLAG RNA was performed as described (Gauczynski *et al*., 2001b). The total volume of the electroporated cells was plated on 10-cm dishes containing 15 ml of complete growth medium followed by incubation for at least 48 h at 37 °C. Fourty-eight hours post transfection, cells were harvested, washed once with phosphate-buffered saline (PBS) and then lysed in PBS supplemented with 0.1% Triton-X100 by repeated freezing and thawing. The crude lysate was obtained by centrifugation at 14 000 r.p.m, 4°C for 15 min and purified by the batch method using an anti-FLAG M2 affinity gel (Sigma). The FLAG-tagged protein was bound over night by rotating at 4°C, washed four times with Tris-buffered saline (TBS), eluted over night by competition with 1 ml TBS containing 100 µg/ml FLAG peptides and dialyzed against 20 mM Hepes, pH 7.4. The purity

and the concentration of the protein was checked by SDS-PAGE followed by silver staining of the gel.

Recombinant proteins generated in the Baculovirus and *Escherichia coli* system

Rec. GST, GST::huPrP23-230, GST::haPrP23-89 and GST::haPrP90-231 were expressed in Baculovirus infected Sf9 cells and purified to homogeneity as described for hamster GST::PrP fusions previously (Weiss *et al*., 1995; Weiss *et al*., 1996). GST-fused PrP-peptides (haPrP23-52, haPrP53-93, haPrP90-109, haPrP129-175, haPrP180-210, haPrP218-231) were expressed in *Escherichia coli* and purified to homogeneity as described for GST fusions (Weiss *et al*., 1995). cDNA encoding for huPrP110-128 was cloned via *Eco*RI (5') and *Bam*HI (3') into pGEX-2T, GST::huPrP110-128 was expressed in *E. coli* and purified to homogeneity as described (Weiss *et al*., 1995). All rec. proteins were dialyzed against 20 mM Hepes, pH 7.4.

PrP-binding assays followed by immunofluorescence analysis

N2a, and human NT2 cells were maintained in DMEM medium containing 10 % FCS, 1 % glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. Mutant CHO cells (S745) as well as wild-type CHO-K1 were cultivated as described above. For competition studies the cells were either pre-incubated for 2 h with the individual antibody diluted in culture medium or co-incubated with rec. protein and antibody (inoculum saturation). In case of pre-incubation, medium was replaced and cells were incubated overnight with 4 µg/ml of rec. GST-fusion proteins per ml of culture medium. Cells were then washed three times with PBS and prepared for immunofluorescence microscopy, which was performed as described (Gauczynski *et al*., 2001b).

PrP-Binding assay in cell culture followed by western blotting

CHO/CHO-S745 cells $8x10^5$ (either non-transfected or transfected with rec. SFV RNAs) were seeded on 6-well plates and incubated at 37 °C. Twenty-four hours post-transfection, cells were incubated in medium containing 5 µg/ml of rec. GST-huPrP23-230 18 h at 37°C. Together with the rec. protein, cells were co-incubated with 40 µg/ml of HSPGs (when indicated). Cells were then washed several times with PBS and scraped off in PBS. After centrifugation the pellets were resuspended in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 1 % NP-40). After addition of Laemmli buffer, samples

were separated by SDS-PAGE and blotted on PVDF membrane. Western blotting was performed with the monoclonal anti-PrP antibody 3B5 or the pAb LRP W3 and peroxidase-coupled secondary antibodies.

Mapping of LRP and PrP-binding sites in the yeast two-hybrid system

Constructions of plasmids pSH2-1 and pJG4-5 were described previously (Rieger *et al*., 1997). For mapping the LRP-PrP interaction site on PrP, the following C-terminal truncated constructs of PrP were generated: pSH2-1-GST::huPrP23-93, pSH2-1-GST::huPrP23-118, pSH2-1-GST::huPrP23-127, pSH2-1-GST::huPrP23-131, pSH2-1-GST::huPrP23-143, pSH2-1-GST::huPrP23-154, pSH2-1-GST::huPrP23-181, and pSH2-1-GST::huPrP180-230. The PrP-fragments were amplified by PCR using oligodesoxyribonucleotides coding for different PrP sequences flanked by a *Bam*HI (5‘) and a *Sal*I (3‘) restriction site. The fragments were cloned via *Bam*HI and *Sal*I into the vector pSH2-1-GST. All PrP constructs were confirmed by sequencing. The different bait plasmids, the prey plasmid pJG4-5-LRP44-295 and the reporter plasmid pSH18-34 (*lac*Z) were co-transformed into EGY48 cells and transformants were tested in β-galactosidase assays**.** Construction of the plasmid pSH2-1-GST::huPrP23-230 was described previously (Rieger *et al*., 1997). For mapping the PrP interaction site on LRP, the C-terminal truncated constructs of LRP pJG4-5-LRP44-101 and pJG4-5-LRP44-160 were designed. The LRP fragments were amplified by PCR using oligodesoxyribonucleotides coding for different LRP sequences flanked by *Eco*RI (5‘) and *Sal*I (3‘). The fragments were cloned via *Eco*RI and *Xho*I restriction sites into the vector pJG4-5. The resulting constructs were confirmed by didesoxysequencing. The different bait plasmids, the prey plasmids and the reporter plasmid pSH18-34 (*lac*Z) were cotransformed into EGY48 cells and transformants were tested in β-galactosidase assays.

LRP-LRP and gal-3-PrP interaction studies in the yeast two-hybrid system

The constructs pSH2-1-GST, pJG4-5-GST, pSH2-1-GST::huPrP and pJG4-5-LRP44-295 were described previously (Rieger *et al*., 1997). The LRP44-295 encoding cDNA was PCR amplified from pJG4-5-LRP44-295. The PCR-product flanked by *EcoRI* (5‘) and *SalI* (3‘) restriction sites, respectively, was cloned into the vector pSH2-1 via both restriction sites resulting in pSH2-1-LRP44-295 which was confirmed by dideoxy sequencing. Yeast transformations and dotting were carried out as described above. Total RNA was isolated from 293 cells by RNeasy kit (Quiagen). Gal-3 cDNA was amplified by RT-PCR and

subcloned into pSH2-1 via *Eco*RI (5‘) and *Sal*I (3‘) restriction sites resulting in pSH2-1-Gal-3, which was confirmed by dideoxy sequencing. The huPrP23-230 encoding cDNA was excised from pSH2-1-GST::huPrP23-230 and subcloned into pJG4-5 via *Eco*RI and *Sal*I. The resulting plasmid pJG4-5-GST::huPrP23-230 was confirmed by didesoxy sequencing. Yeast transformations and dotting was carried out as described above.

Analysis of native LRP::FLAG by size-exclusion chromatography

The Superose 12 PC 3.2/30 column (Amersham Pharmacia) was calibrated with the LMW calibration kit in 20 mM HEPES pH 7,4. Purified LRP-FLAG (2.5 µg) of expressed in the SFV system were loaded in a total volume of 25µl. Chromatography was performed at a flow rate of 30µl/min. The eluted LRP was detected with a UV-M II monitor at 280 nm.

***In vitro* interaction of PrP and LRP**

Rec. FLAG::huPrP23-230 was expressed in the Baculovirus system (C. Hundt *et al*, manuscript in preparation) according to rec. FLAG::haPrP23-231 (Rieger *et al*., 1997) and immobilized on anti-FLAG M1 beads. Twenty microlitres of beads (1:1 slurry; 500 ng of FLAG::huPrP), were incubated in TBS supplemented with 2 mM $MgCl_2$ with 500 ng of rec. GST and 1 µg of GST-LRP (Rieger *et al*., 1997), respectively (molar ratio: 1:1) in the absence and presence of 1.5 µg HSPGs (Sigma). Homogeneity of GST-LRP, GST and FLAG::huPrP23-231 was proven on silver-stained SDS-PA gels. After 1h at room temperature, the supernatant was removed, beads washed four times with TBS, boiled in SDS-sample buffer and analysed by western blotting developed with mAb GST.

Antibodies

pAb LRP W3 (Rieger *et al*., 1997) was purified by protein A-Sepharose chromatography. mAb GST (Santa Cruz Biotechnology), mAb VLA6 (Immunotech.), mAb 3B5 (G. Hunsmann), mAb SAF70 (aa 140-180 of PrP) and pAb JB007 (CEA, France), secondary fluorescein isothiocyanate (FITC), Cy3 (indocarbocyanine) and Texas Red-conjugated antibodies (used at 1:100 dilutions; Jackson Laboratories/Southern Biotechnology) were used.

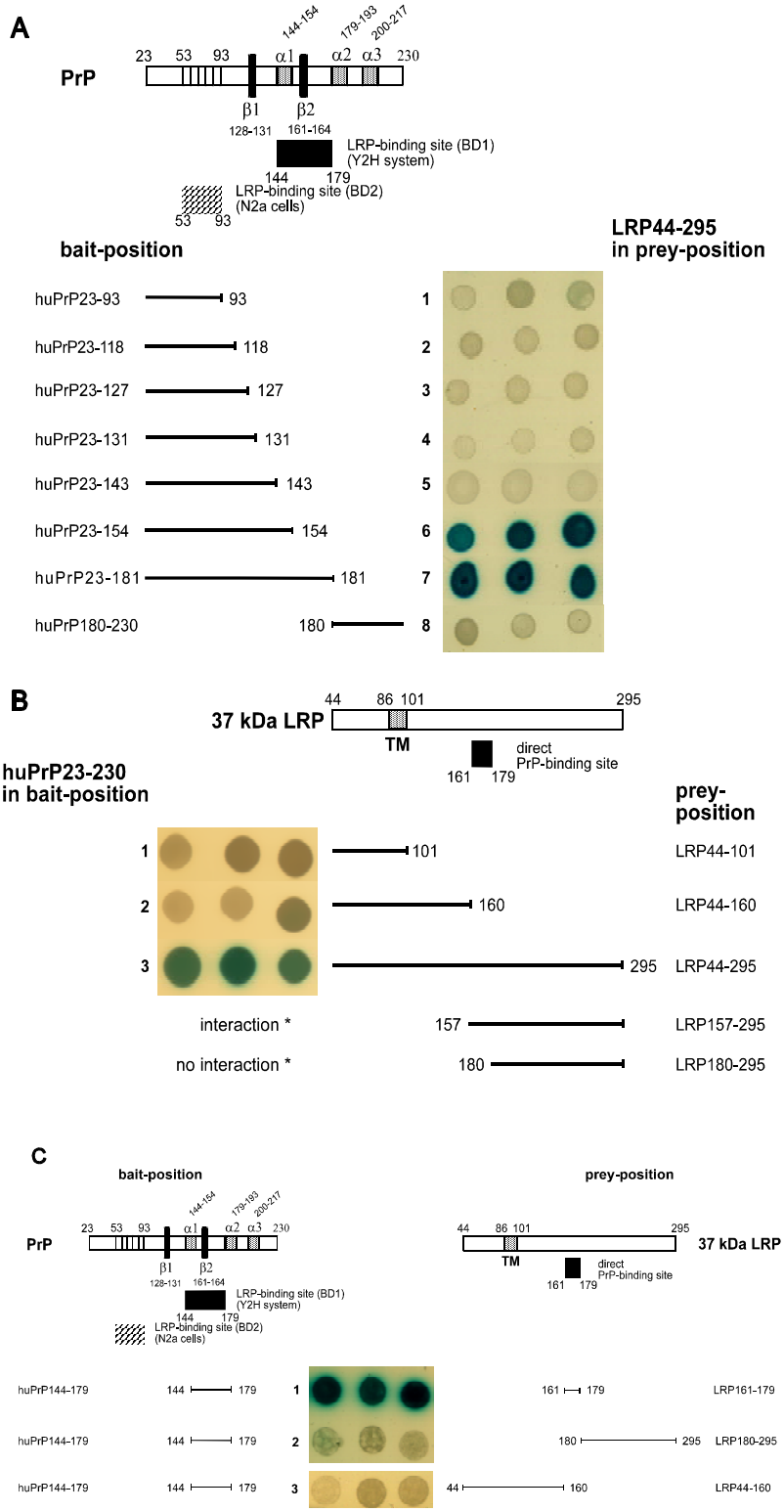


Fig. 1. Identification of direct PrP-LRP interaction domains. (**A**) Identification of the direct PrP-LRP interaction domain on PrP. HuPrP23-93 (row 1), huPrP23-118 (row 2), huPrP23-

127 (row 3), huPrP23-131 (row 4), huPrP23-143 (row 5), huPrP23-154 (row 6), huPrP23-181 (row 7) and huPrP180-230 (row 8) were co-expressed in fusion with GST in the bait position together with LRP in prey position of the yeast two-hybrid system. (**B**) Retrenchment of the direct PrP-LRP interaction domain on LRP (Rieger *et al*., 1997). LRP44-101 (row 1), LRP44-160 (row2), LRP44-295 (row 3) were co-expressed in prey position together with huPrP23-230 fused to GST in bait position of the yeast two-hybrid system. *Interactions between LRP157-295 and LRP 180-295 (in prey position) versus huPrP23-230 fused to GST (in bait position) have been investigated previously (Rieger *et al*., 1997). (**C**) PrP144-179 interacts directly with LRP161-179 in the yeast two-hybrid system. PrP144-179 fused to GST in bait position was co-expressed with LRP161-179 (row 1), LRP180-295 (row 2) and LRP44-160 (row 3) in prey position of the yeast two-hybrid system. (A-C) All interactions were monitored by the β-galactosidase reporter system.

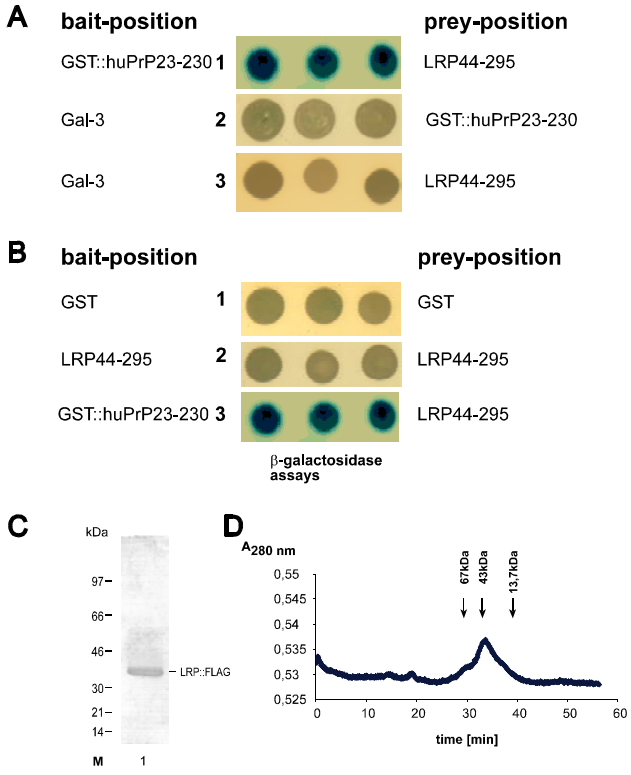


Fig.2. LRP fails to interact with itself in the yeast two-hybrid system and appears monomeric by native size-exclusion chromatography. Gal-3 fails to interact with PrP and LRP. (**A**) huPrP23-230 and LRP fail to interact with the β-galactoside lectin gal-3 in the yeast two-hybrid system. huPrP23-230 fused to GST in bait position was co-expressed with LRP44-295 in prey position (row 1), gal-3 in bait position was co-expressed with GST::huPrP23-230 (row 2), and LRP44-295 (row 3) in prey position. **(B**) LRP fails to interact with itself in the yeast two-hybrid system. GST (row 1), LRP44-295 (row 2) and GST::huPrP23-230 (row 3) were expressed in bait and GST (row 1), and LRP44-295 (row 2 and 3) in the prey position of the yeast two-hybrid system. Detection (A and B) by the β-galactosidase reporter system. (**C**) Analysis of rec. LRP::FLAG on SDS-PAGE. One microgram of rec. LRP::FLAG purified under native conditions from the SFV system was analyzed on a 12.5 % SDS-PA-gel stained with silver (lane 1). Marker proteins are indicated. (**D**) Analysis of rec. native LRP::FLAG by size exclusion chromatography. Homogeneous LRP::FLAG (2.2 µg) were analyzed by size

exclusion chromatography on a Superose12PC3.2/30 column (Amersham Pharmacia). Marker proteins are indicated.

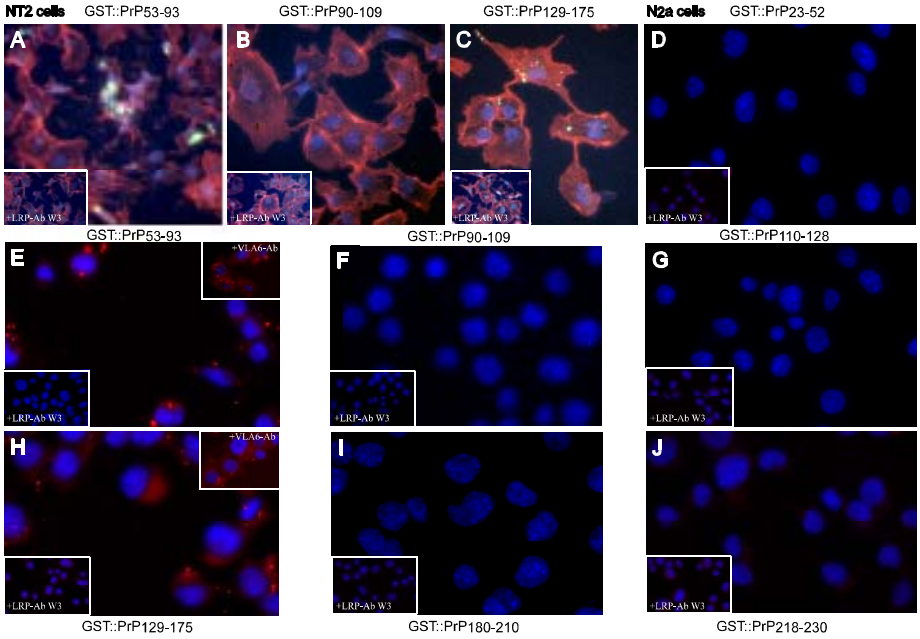
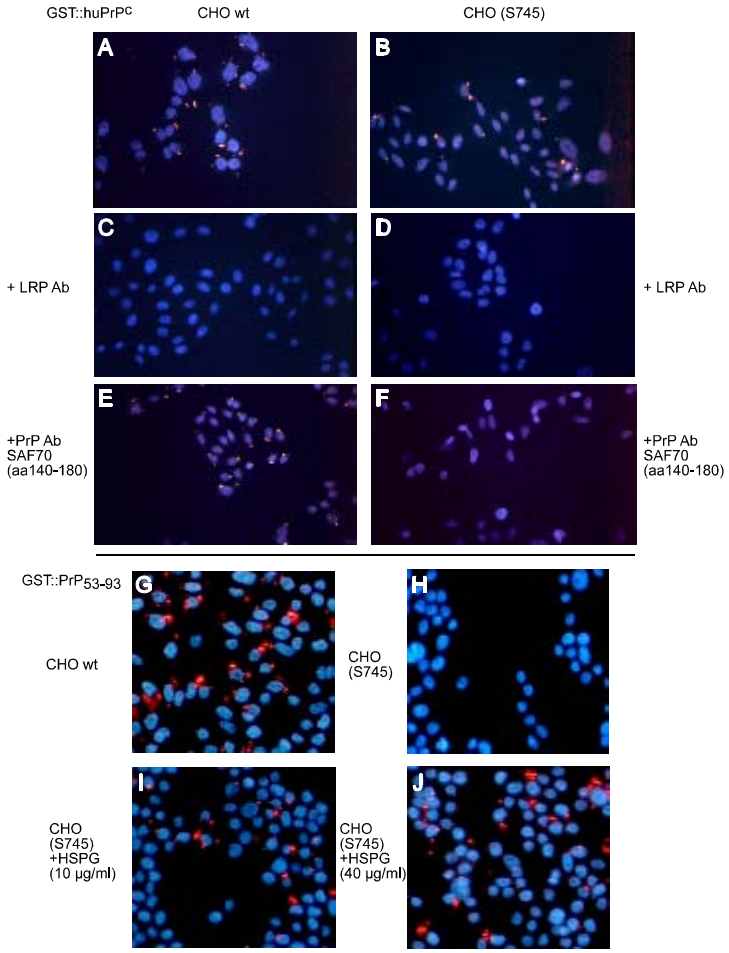


Fig. 3. Identification of PrP-interaction domains for LRP/LR by binding assays with rec. prion peptides on NT2 and N2a cells. (A-C) Binding to NT2 cells. NT2 cells were incubated with PrP peptides fused to GST in the absence (A-C) and presence (insets in A-C) of the preincubated pAb LRP W3 (dilution 1:50). The following peptides (4 µg/ml) were used: GST::PrP53-93 (**A**), GST::PrP90-109 (**B**) and GST::PrP129-175 (**C**). Immunofluorescence analysis was performed by triple labelling involving actin staining (phalloidin, red), nuclear staining [4‘,6-diamidino-2-phenylindole (DAPI), blue] and GST staining (sec. Ab FITC, green) (magnification x630). (**D-J**) Binding to N2a cells. N2a cells were incubated with PrP peptides fused to GST in the absence (D-J) or presence of either pre-incubated pAb LRP (dilution 1:50) (bottom insets in D-J) or pre-incubated mAb VLA6 (dilution 1:50) (top inset in E and H). The following peptides (4 µg/ml) were used: GST::PrP23-52 (**D**), GST::PrP53-93 (**E**), GST::PrP90-109 (**F**), GST::PrP110-128 (**G**), GST::PrP129-175 (**H**), GST::PrP180-210 (**I**) and GST::PrP218-230 (**J**). Immunofluorescence was performed with mAb GST, sec. Ab Texas Red, DAPI staining; magnification x400).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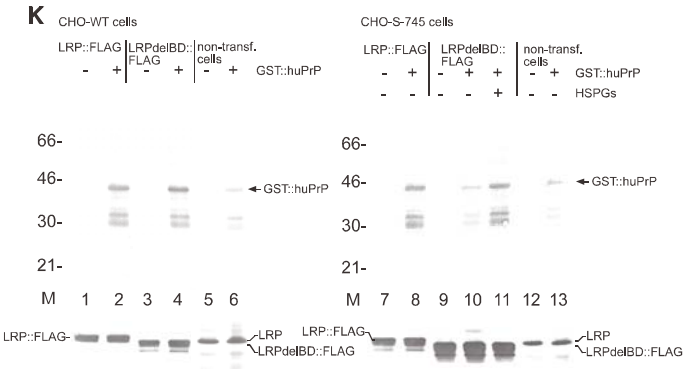
CHO wt
CHO (S745)
A
B
C
D
+ LRP Ab
+ LRP Ab
E
F
+PrP Ab SAF70 (aa140-180)
+PrP Ab SAF70 (aa140-180)
GST::PrP53-93
G
H
CHO wt
CHO (S745)
I
J
CHO (S745) +HSPG (10 µg/ml)
CHO (S745) +HSPG (40 µg/ml)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non-transf. cells
GST::huPrP
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LRP::FLAG
LRP
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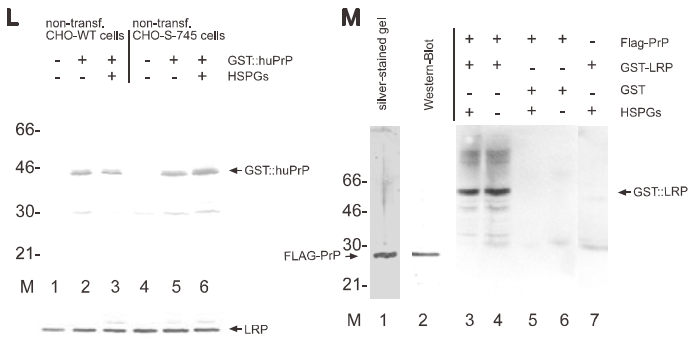
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HSPGs
66-
46-
30-
21-
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LRP
M
silver-stained gel
Western-Blot
Flag-PrP
GST-LRP
GST
HSPGs
GST::LRP
FLAG-PrP
M 1 2 3 4 5 6 7

Fig. 4. Influence of HSPGs on the LRP-LR-PrP-binding reaction analyzed by wild-type and mutant HSPG-deficient CHO cells; use of an LRP deletion mutant lacking the direct PrP-binding domain on LRP; PrP-LRP *in vitro* interaction studies. (A-F) Binding of GST::huPrP23-230 to CHO cells and to HSPG-deficient CHO cells (S745) (Esko *et al*., 1985) in the absence and presence of antibodies. Binding of 4 µg/ml GST::huPrP23-230 to CHO wild-type cells in the absence of any antibody (**A**), in the presence of the pAb LRP W3 (1:50) (**C**), mAb SAF 70 (aa140-180 of PrP) (**E**). Binding of GST::huPrP23-230 to CHO-S745 cells in the absence of any antibody (**B**), in the presence of the pAb LRP W3 (**D**) and in the presence of the mAb SAF 70 (**F**). The mAb SAF70 was used at a 1:1000 dilution to saturate GST::huPrP23-230 while pAb LRP was pre-incubated with the cells at a 1:50 dilution prior to addition of the rec. PrP. Immunofluorescence analysis: pAb LRP (sec. Ab FITC), mAb 3F4 (sec. Ab Texas Red; DAPI staining; magnification 400x). (G-J) Binding of GST::PrP53-93 to CHO wild-type and CHO-S745 cells. Binding of 4 µg/ml GST::huPrP53-93 to CHO wild-type (**G**) and CHO-S745 cells in the absence of HSPGs (**H**), and in the presence of 10 (**I**) and 40 µg/ml (**J**) HSPGs, respectively. Immunofluorescence analysis: mAb GST (sec. Ab Texas Red; DAPI staining; magnification x400). (**K**) Binding of PrP by wild-type CHO and mutant HSPG-deficient CHO-S745 cells hyperexpressing LRP::FLAG or LRPdelBD::FLAG (lacking aa 161-180). CHO cells (lanes 1-6) either hyperexpressing LRP::FLAG (lanes 1 and 2), LRPdelBD::FLAG (lanes 3 and 4) by the SFV system or non-tranfected (lanes 5 and 6) were incubated with 5 µg/ml GST::huPrP (lanes 2, 4 and 6). CHO-S745 cells (lanes 7-13) either hyperexpressing LRP::FLAG (lanes 7 and 8), LRPdelBD::FLAG (lanes 9-11) by the SFV system or non-transfected (lanes 12 and 13) were incubated with 5 µg/ml GST::huPrP (lanes 8, 10, 11 and 13). HSPGs (40 µg/ml) were added simultaneously with GST::huPrP to the CHO-S745 cells overexpressing LRPdelBD::FLAG (lane 11). Total cell extracts were analyzed by western blotting employing the mAb 3B5 (upper panels) or pAb LRP W3 (lower panels). (**L**) Binding of PrP by non-transfected wild-type CHO and mutant HSPG-deficient CHO-S745 cells. Non-transfected CHO wild-type cells (lanes 1-3) and non-transfected CHO-S745 cells (lanes 4-6) were incubated with 5 µg/ml GST::huPrP (lanes 2, 3, 5 and 6). HSPGs (40 µg/ml) were added simultaneously with GST::huPrP to both cell types (lanes 3 and 6, respectively). Total cell extracts were analyzed as described in (K). Interaction of rec. FLAG::PrP and rec. GST::LRP *in vitro* (**M**). FLAG::huPrP23-230 (0.5 µg) immobilized on anti-FLAG Sepharose beads analyzed on a 12 % SDS-PA gel stained with silver (lane 1) and by western blotting employing the pAb JB007 (lane 2) were incubated with 1 µg of GST::LRP in the absence (lane 4) or in the presence of 1.5 µg/µl HSPGs (lane 3), 0.5 µg of GST in the absence (lane 6) or the presence of 1.5 µg/µl HSPGs (lane 5). Unloaded beads were incubated with 1 µg of GST::LRP in the presence of 1.5 µg/µl HSPGs (lane 7). Beads after washing were analyzed by western blotting on a 12% SDS PA-gel employing mAb GST (sec. antibody POD).

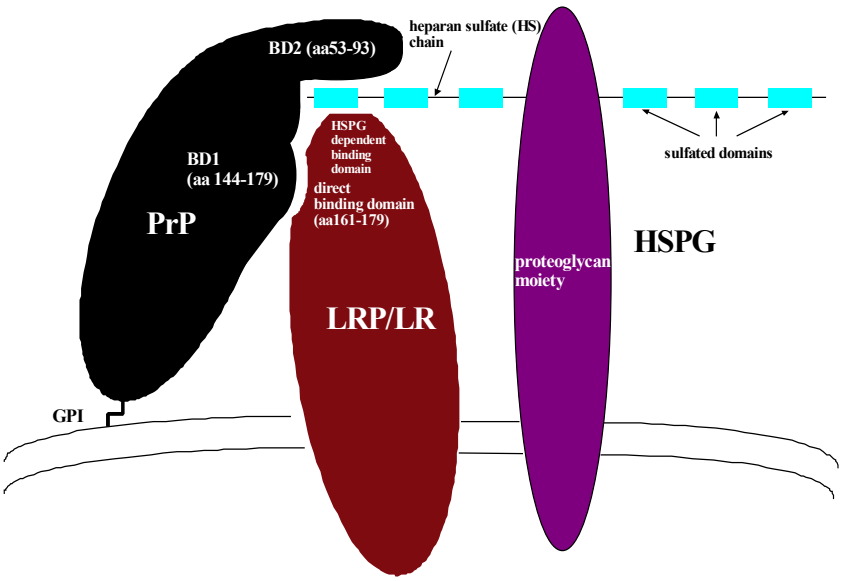

Fig. 5. Model for the function of LRP-LR as the receptor for PrP. The PrP molecule binds to LRP-LR via PrP^{LRPbd1} and PrP^{LRPbd2}. PrP^{LRPbd2} (aa 53-93) is dependent on the presence of a heparan sulfate arm of a HSPG molecule whereas PrP^{LRPbd1} (aa 144-179) interacts directly with LRP-LR (as shown in the yeast two-hybrid system). The simultaneous presence of both PrP^{LRPbd1} and PrP^{LRPbd2} would stabilize considerably the binding of the entire PrP molecule to its receptor. Direct binding of LRP-LR to PrP occurs via the direct binding site located between aa 161-179 of LRP-LR (Figure 1). The indirect HSPG-dependent binding domain might locate between aa 101 and 160 or between aa 180-285 [presumably aa 205 and 229 of LRP (Kazmin *et al*., 2000)]. The association of LRP-LR with HSPGs might explain the relationship between 37-kDa LRP and 67-kDa LR.

Chapter VI

Identification of the Heparan Sulfate Binding Sites in the Cellular Prion Protein

submitted for publication:
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Abstract

Data from cell culture and animal models of prion disease support the separate involvement of both heparan sulfate proteoglycans and copper (II) ions in prion (PrP) metabolism. Though direct interactions between prion protein and heparin have been recorded, little is known of the structural features implicit in this interaction or of the involvement of copper (II) ions. Using biosensor and ELISA methodology we report direct heparin and heparan sulfate-binding activity in recombinant PrP^{c}. We also demonstrate that the interaction of recombinant PrP^{c} with heparin is weakened in the presence of Cu(II) ions and is particularly sensitive to competition with dextran sulfate. Competitive inhibition experiments with chemically modified heparins also indicate that 2-*O*-sulfate groups (but not 6-*O*-sulfate groups) are essential for heparin recognition. We have also identified three regions of the prion protein capable of independent binding to heparin and heparan sulfate: residues 23-52, 53-93 and 110-128. Interestingly, the interaction of an octapeptide-spanning peptide motif aa53-93 with heparin is enhanced by Cu(II) ions. Significantly, a peptide of this sequence is able to inhibit the binding of full-length prion molecule to heparin, suggesting a direct role in heparin recognition within the intact protein. The collective data suggest a complex interaction between prion protein and heparin/ heparan sulfate and has implications for the cellular and pathological functions of prion proteins.

Introduction

Considerable effort has been devoted to the identification of natural receptors for prion protein (PrP) both to refine the understanding of prion metabolism and to reveal potential targets for therapeutic intervention in the transmissible spongiform encephalopathies (TSEs). One group of potential co-factors are the glycosaminoglycans (GAGs) and in particular membrane proteins and extracellular matrix components elaborated with heparan sulfate (HS) sugar chains. Heparan sulfates (of which heparin is a heavily sulfated variant) are expressed on a wide variety of cell types, including those of neural origin, and modulate the activity of a wealth of cell-surface and extracellular signaling molecules such as growth factors and cytokines (Maccarana *et al*., 1993; Wrenshall and Platt, 1999). In the TSE field it is known that HSPGs co-localize with the insoluble aggregates of the prion (PrP^{Sc}) that accumulate in the brain tissue of TSE affected animals (Guiroy *et al*., 1991; McBride *et al*., 1998; Snow *et*

al., 1990). They also promote the formation of amyloid structures typical of Alzheimer's disease (a condition with many morphological similarities to prion diseases) when co-injected with Aβ-protein into the brains of rats (Snow *et al*., 1994). Administration of selected anionic compounds can restrict tissue-specific accumulation of PrP^{Sc} and the onset of neurodegenerative features in experimental models of prion disease, Pentosan sulfate and DS500 (dextran sulfate with av. MW 500,000) being particularly effective examples (Ladogana *et al*., 1992; Beringue *et al*., 2000). The mode of action of these compounds is uncertain, one possibility being that they compete with endogenous HSPGs for prion. A direct association with amyloid structures has also been considered (Caspi *et al*., 1998). Sulfated GAGs and lipopolyamines (a group of molecules that reverse many of the actions of heparin) modulate the expression of PrP^{Sc} in cultured cells (Caughey and Raymond, 1993; Gabizon *et al*., 1993; Supattapone *et al*., 1999) and several investigators have described direct interactions between cellular or recombinant prion and GAGs (Brimacombe *et al*., 1999; Caughey *et al*., 1994). Of particular significance is the detection of a glucose polysaccharide in prion rods purified from scrapie-infected hamster brains (Appel *et al*., 1999). Two recent papers suggest that HS may play a critical role in the molecular events leading to PrP^{sc} production and the acquisition of infectivity. Wong and colleagues have demonstrated that the cell-free conversion of PrP to a protease-resistant folding-variant may be stimulated by the addition of HS (Wong *et al*., 2001) and an heparitinase-sensitive fraction of cell extract will promote the reconstitution of infectivity to DMSO-dispersed prion rods (Shaked *et al*., 2001a).The 37 kDa laminin receptor (LRP/LR), identified originally as a potential binding partner for PrP (Rieger *et al*., 1997) has now been confirmed as a cell surface receptor (Gauczynski *et al*., 2001) and heparan sulfate proteoglycans (HSPGs) have been identified as co-factors in this interaction via indirect binding domains on both proteins (Hundt *et al*., 2001).

A notable feature of prion biology is the chelation of divalent cations, especially copper (Brown *et al*., 1997; Hornshaw *et al*., 1995; Jobling *et al*., 2001), and there is considerable interest in the possible connection between metal-binding status and conformation (Brown *et al*., 2000; Miura *et al*., 1999; Qin *et al*., 2000; Shaked *et al*., 2001b; Wong *et al*., 2000). Metal binding resides primarily in an octapeptide repeat motif between residues 53-93 of mammalian PrP though more distal binding sites have also been proposed (Jobling *et al*., 2001; Shaked *et al*., 2001b). Intriguingly manganese and copper have quite distinct effects on the adoption of proteinase resistance by PrP (Brown *et al*., 2000). The role of this octapeptide repeat in prion disease progression is poorly understood. Though PrP null-mutant mice can be

restored to scrapie susceptibility by introduction of PrP transgenes lacking this motif (Fischer *et al*., 1996; Flechsig *et al*., 2000), scrapie incubation times are still extended compared to wild type (Supattapone *et al*., 2001).

Combined heparin-binding activity and divalent cation chelation have been demonstrated for several other proteins, notably superoxide dismutase (Marklund, 1982) and the APP protein of Alzheimer's disease (Multhaup *et al*., 1995). A potential interaction between the heparin- and copper-binding functions of prion protein would be an intriguing scenario. There is certainly evidence that the two functions map to the same region of the molecule. Shyng and colleagues have reported that the region between residues 25 and 91 (incorporating the octapeptide repeat region) is sufficient for PrP binding to HSPG moieties on N2a cells. They also demonstrated that unlike intact PrP^{c}, the surface expression of a mutant PrP without this N-terminal segment is not subject to down-regulation by provision of soluble pentosan sulfate (Shyng *et al*., 1995). Elsewhere, a naturally truncated PrP present in significant amounts in the human brain and lacking residues amino-terminal to 111 or 112 (including the octapeptide-repeat), has no heparin-binding activity (Chen *et al*., 1995). Furthermore the PrP sequence aa53-93 is directly involved in an HSPG-dependent interaction between recombinant PrP and laminin receptor precursor (LRP) (Hundt *et al*., 2001). Significantly, whereas Caughey found that heparin binding by PrP^{c} is independent of divalent ion concentration (Caughey *et al*., 1994) Brimacombe has reported a study in which the binding of recombinant PrP to experimental nickel surfaces is heparin-sensitive (Brimacombe *et al*., 1999).

The only other region of PrP with supporting evidence for a role in GAG binding is the central, hydrophobic and amyloidogenic sequence between residues 106-126 (De Gioia *et al*., 1994; Gasset *et al*., 1992). This sequence undoubtedly plays a major role in the biosynthesis of the protease-resistant form of PrP (PrP^{Sc}). Cells expressing engineered variants of PrP^{c} deleted for residues in this region of the molecule do not support the propagation of homologous PrP^{Sc} and PrP molecules deleted for this sequence cannot be converted to protease-resistant forms *in vitro* (Horiuchi and Caughey, 1999). Several groups have investigated the cytotoxic properties of the central region (Forloni *et al*., 1993; Jobling *et al*., 1999). Significantly a neurotoxic activity associated with a peptide corresponding to residues 106-126 can be abrogated by soluble heparin and related GAGs (Perez *et al*., 1998). Though copper has been shown to affect the aggregation and neurotoxic properties of this region (Jobling *et al*., 2001), the influence of copper on any interaction with GAGs is not documented.

Recombinant PrP has now been expressed and purified by several groups (Volkel *et al*., 1998; Weiss *et al*., 1995; Zahn *et al*., 1997). These efforts have yielded proteins that resemble PrP^c in that they fold into α-helical and β-sheeted structures (Gauczynski *et al*., 2001; Volkel *et al*., 1998). They are also proteinase K sensitive (Weiss *et al*., 1996), suggesting a lack of infectivity. Crucially, N-terminally-tagged PrP retains propagating activity (Telling *et al*., 1997) and PrP retains Hsp60 chaperone binding activity when tagged with GST (Edenhofer *et al*., 1996).

In this study we confirm a direct interaction between recombinant PrP^c and both heparin and HS and show that the binding of full-length recombinant GST::PrP^c to heparin is significantly weakened in the presence of copper (II) ions. Competitive inhibition studies reveal that dextran sulfate is a highly potent inhibitor of the PrP^c-heparin interaction, and that 2-*O* sulfates of heparin are an essential component of the PrP^c binding site(s). In a second series of experiments biosensor and ELISA analysis applied to both glutathione sulfotransferase-tagged recombinant peptides (covering the whole sequence of hamster PrP; (Hundt *et al*., 2001; Edenhofer *et al*., 1996)) and synthetic peptides have enabled us to identify three sequences in PrP with independent heparin / HS binding activity: residues 23-52, 53-93 and 110-128. At higher concentrations than those which affect intact PrP protein, copper (II) enhances heparin-binding by isolated peptide 53-93, and in the presence of Cu(II) this peptide effectively competes with full-length haPrP^c for heparin binding. The divergent actions of copper (II) ions on full-length PrP and on the peptide 53-93 are discussed.

Experimental procedures

Materials

Bovine lung heparin (BLH; H-4898), porcine intestinal heparin (PIH; H-9399), low molecular weight heparin from porcine intestinal mucosa (LMW PIH; H-5284, average mol. weight approx. 6000), pentosan polysulfate (PPS), dextran sulfate (DS8; D-4911, average mol. weight approx. 8000) and chondroitin sulfate (CS; C-8254) were obtained from Sigma. Porcine Intestinal heparan sulfate fraction II (PMHS) was obtained from Organon and bovine kidney HS (BKHS, H-7640) was from Sigma. Both types of HS were pre-treated with chondroitin ABC lyase to eliminate chondroitin sulfate contaminants. Stock solution of 2.2 mM Cu(II), Zn(II), Ni(II), Mg(II) and Mn(II) were prepared in glycine (4.5 mM in distilled water) after the method of Brown *et al*. (Brown *et al*., 1997). Recombinant streptavidin (S-

0677) from *streptomyces avidinii* was purchased from Sigma. The biotin donors biocytin hydrazide (B-9014), biotin amidocaproate-NHS (B-3295) and biotin amidocaproate 3-sulfo-NHS (B-1022) were also from Sigma. Modified heparins: (persulfated (oversulfated), and 2*O*- and 6-*O*-desulfated BLH) were prepared and sulfation status confirmed as previously reported (Jaseja, 1989; Yates *et al*., 1996).

Expression and purification of recombinant hamster GST::PrP^c fusion protein and peptides

GST::PrP^c (23-231) of Syrian golden hamster and human sequence and the GST::haPrP^c fragments GST::P23-52 (P1), GST::P53-93 (P2), GST::P90-109 (P3), GST::P110-128 (P4n), GST::P129-175 (P4), GST::P180-210 (Px) and GST::P218-231 (P5) were expressed from baculovirus in Sf9 insect cells (GST::PrP^c) and from *E. coli* (peptides) as previously described (Hundt *et al*., 2001; Weiss *et al*., 1995). Protein and peptides were dialysed into 20mM HEPES pH 7.4 and stored at 4^{o}C. Heparin/ HS binding activity was examined within 2 months of preparation.

Preparation of synthetic PrP peptides with human sequence

Peptides corresponding to residues 23-52 (P1), 53-93 (P2), 90-109 (P3), 110-128 (P4n), 129-175 (P4), 180-210 (Px) and 218-231 (P5) of the normal human PrP sequence were synthesized on an AMS 422 multiple peptide synthesizer (Abimed) using FMOC chemistry (preloaded HMP (Wang) resin, tBu/Trt protection, Arg-Pmc; Trp-Boc, PyBOP activation). After completion of the synthesis the peptides were cleaved using 92.5% TFA / 2.5 % H2O / 5% tri-Isopropylsilane, precipitated and washed with tert-Buthyl-methyl-ether. The deprotected crude peptides were purified by reversed-phase HPLC (Sykam HPLC-system, GROM C18 column, 20 x 250 mm, 5 µ). The identity of the purified peptides was confirmed by MALDI-TOF mass spectrometry (Bruker Reflex III).

Biotinylation of heparin and HS for immobilisation

Three methods were adopted for biotinylation of heparins and HS: a.) GAGs were labeled by reaction of their aldehydic reducing groups using a method based on Nadkarni *et al.* (Nadkarni and Linhardt, 1997) Briefly 50 nmol of saccharide was dissolved in 50 µl formamide containing 50 mM biocytin hydrazide and heated at 37^{o}C for 24 h. Heparin/ HS labeled in this way was used for occasional biosensor analysis of GST::haPrP^c binding. b.) The second procedure is a modification of that described by Rahmoune *et al*. (Rahmoune *et*

al., 1998) and labels the free amino groups reported to occur occasionally along the length of heparin and HS. To 50 nmol heparin / HS in dH_2O was added 30 µl of a 50 mM solution of biotin amidocaproate-NHS in DMSO. The mixture was briefly mixed and left for 3 days at room temperature. This method was used to biotinylate heparin and HS for biosensor studies of both GST::haPrP^c and peptide binding. c.) A third procedure, used in ELISA studies of PrP and peptide binding is a modification of that of Lee and Conrad and also labels mid-chain (Lee and Conrad, 1984): 5 µmol heparin or HS was dissolved in 0.5 ml of sodium carbonate buffer pH 8.6 containing 15 µmol biotin amidocaproate 3-sulfo-NHS. The mixtures were shaken briefly to mix and left to stand at room temperature for 3 days. Free label and solvent was removed from all labelling reactions as follows: 5 volumes of pre-chilled ethanol were added to each tube and the sample stored at –20^o C for 30 minutes. The sample was next centrifuged (5 minutes, 13,000 rpm) and the ethanol decanted, chilled for a second time and re-centrifuged. The precipitate from both stages was combined in 400µl dH_2O and fractionated by gel filtration using 3 x Hi-Trap columns (Pharmacia) arranged in series. Biotin-containing fractions were detected at 232 nm and the labeled GAG eluting in the void volume was retained.

Biosensor analysis of GST::PrP^c and PrP peptide binding to heparin and HS

Biosensor analysis was performed on a Bia2000 instrument (BiaCore). Two channels of a Pioneer-C1 biosensor chip (BiaCore, planar surface) were coated with streptravidin (injection of 50 µl solution of streptavidin, 1 mg/ml in sodium acetate buffer, pH 4.5). One streptavidin conditioned channel was then incubated with biotinylated heparin or HS (method of biotinylation dependent on test protein (see above), and the second surface left unmodified to control for non-GAG specific binding events. The mobile phase in all biosensor analyses was HBS-P (HEPES buffered saline, 10mM HEPES pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20) pre-prepared by the manufacturer (BiaCore). GST::ha PrP^c and GST::ha PrP^c partial peptides were obtained as dilute (ca. 10-100 ng/µl) solutions in 20mM HEPES buffer, pH 7.4. Proteins were injected with no additional dilution, or pre-diluted in HBS-N (HEPES buffered saline with no detergent). All samples were dispensed in microfuge tubes and centrifuged for 5 minutes at 10000 rpm prior to analysis to remove particulates. Samples were injected (KINJECT command, 30 µl) onto both biosensor surfaces at a flow-rate of 10-20 µl/min and at a temperature of 25 ^{o} C. A dissociation period of 120 seconds was selected. The following solutions were routinely used to regenerate the chip surfaces between PrP samples (volume): 2M NaCl (10 µl), 10mM HCl (5 µl) 10 mM NaOH (5 µl) and 2 mg/ml BLH (10 µl). To

resolve heparin/HS specific binding events the pattern of mass changes (response units: RU) recorded at the un-derivatised surface of ther biosensor chip was subtracted from the signal recorded at the heparin/ HS-derivatised surface.

ELISA analysis of binding of GST::PrP peptides and protein to heparin

Analysis of heparin /HS binding by full-length and peptidic GST fusions was performed on Maxisorb and Polysorb (Nunc) 96-well plates respectively. The plates were pre-coated with 3 μg/ml streptavidin in 0.2 M bicarbonate buffer, 0.15 M NaCl, pH 9.3 and left to stand overnight at 4^{o}C. Plates were then washed briefly in PBST (0.05% Tween-20 in phosphate buffered saline, pH 7.4) and blocked for 2 hours with 10% Seablock blocking reagent (Pierce), 0.5% Tween-20 in phosphate buffered saline. Biotin-conjugated bovine lung heparin, porcine intestinal heparin and porcine mucosal HS substrates were then applied to the plates (3 columns each x 8 rows) at a concentration of 75 μg/ml diluted in PBST. A separate array of wells (3 columns x 8 rows) was incubated with PBST alone. Biotinylated heparin / HS / PBST was left in contact with the wells for a minimum of three hours at room temperature after which period the wells were washed with PBST. GST-tagged proteins were the applied, diluted in 10% Seablock (Pierce) in PBS to minimise non-specific interactions. In general, one row of 12 wells (3 wells each per biotinylated GAG and control) were devoted to each GST::PrP^{c} or GST::peptide preparation, and 35 μl of sample dispensed in each well. In certain experiments PrP preparations were mixed with non-biotinylated GAGs and synthetic PrP peptides prior to incubation. Divalent cations ions were also supplemented in certain experiments, from stock mixtures with glycine. Following the incubation period (minimum 2 hour duration) GST::PrP^{c} / peptide was decanted and the wells washed thoroughly with PBST. All wells were then treated (1hr at room temperature) with 35 μl of either monoclonal anti-GST (Clone GST-2, G-1160, Sigma) or polyclonal rabbit anti-GST (G-7781, Sigma) applied at a dilution of 1:200 in PBST. In a final incubation phase, after another round of washing, wells were incubated (1 hour, room temperature) with 35 μl sheep anti-mouse IgG (F(ab')^{2})-peroxidase conjugate (NA 9310, Amersham, for assays employing the monoclonal anti-GST) or anti-rabbit Ig (F(ab')^{2})-peroxidase conjugate (NA-9340, Amersham, for assays employing the polyclonal anti-GST) diluted 1:1000 in PBST. Colour was developed by addition of 100 μl per well *o*-phenylene-diamine substrate solution (1 x 30mg OPD tablet (Sigma P-8412) per 75 μl phosphate-citrate buffer (0.05M) pH 5.0 containing 0.03% sodium perborate (Sigma P-4922)). Absorbance was measured at 490nm after quenching the wells

with 50 µl 0.5M H_2SO_4. Results with monoclonal and polyclonal anti-GST antibodies were essentially identical.

ELISA analysis of binding of GST::haPrP^c to immobilised synthetic PrP peptides.

An ELISA was established to examine the potential for PrP-PrP interactions. Peptides P1 (23-52), P2 (53-93) and P4n (110-128) (50 µg/ml in 0.2 M sodium bicarbonate buffer, pH 9.3 containing 0.15 M NaCl) were coated onto wells of a Maxisorb microtitre plate (Nunc). A fourth block of three columns was left uncoated. After coating (overnight, 4^{o}C) all wells were blocked with 3% bovine serum albumin in PBS containing 0.2% tween-20 (2 hours at room temperature). GST::haPrP^c was subsequently applied at 1µg/ml in PBS containing 3% BSA. After 2 hours incubation the plate was thoroughly washed (6 x in PBS/ 0.05% tween-20) and bound GST::haPrP^c detected with polyclonal rabbit anti-GST/ anti-rabbit Ig (F(ab')^2)-peroxidase conjugate as described above (conventional heparin-binding ELISA). In inhibition studies a selection of GAGs (100 µg/ml) were supplemented during the GST::haPrP^c incubation phase.

Results

Recombinant hamster PrP^c binds to immobilised heparin and HS

Surface plasmon resonance instruments are able to detect the mass changes that accompany the binding of soluble analyte to ligand immobilised on a detector surface. Initial experiments in this study investigated the binding of the GST::haPrP^c fusion protein to immobilised heparin and HS. Figures 1a and 1b show the resonance profiles or sensorgrams recorded when short pulses of recombinant hamster GST::PrP^c were injected over bovine lung heparin and porcine intestinal HS surfaces (immobilised via mid-chain biotinylation). The sensorgrams indicate the net binding response on the derivatised surfaces after subtraction of any signal generated on an underivatised control surface. Also shown are the resonance patterns generated by injection of an identical volume of carrier buffer alone ("buffer"). During the period of contact between sample and surface (phase A, 180 secs), a positive binding signal which was considerably higher than that of buffer alone (60-80 RU *vs.* 10 RU) was observed on both GAG-derivatised surfaces. Since the size of the signal achieved is in part dependent on the density of immobilised heparin and HS (unknown for these surfaces) it is not appropriate to estimate realtive binding avidity to heparin and HS from a comparison of signal

strengths. Under identical conditions glutathione sulfotransferase alone yielded no net binding to heparin or HS-derivatised surfaces. The two surfaces differed considerably in the shape of the response curve during the dissociative period (D, 120 secs). Approximately half of the GST::haPrP^{c} which had accumulated on the heparin surface during the contact phase (A) showed very rapid dissociation at the onset of the dissociative phase (D) to be followed by a stable phase when very little further movement was recorded. In contrast dissociation of GST::haPrP^{c} from the HS surface was more progressive, though the proportion of accumulated RU lost in the dissociative phase was similar to heparin at 50 percent. Both surfaces were washed with short pulses of strong saline (2M NaCl) and weak acid (10mM HCl) at identical times after PrP application. These washes would be expected to have a strongly disruptive effect on heparin and HS directed binding. Such washing accounted for approximately 50% and 70% removal of residual GST::haPrP^{c} from heparin and HS respectively.

Prion protein is known to bind copper (II) ions *in vitro*, and earlier investigations have revealed a linkage between divalent cation and polyanion binding activities (Brimacombe *et al*., 1999). This prompted us to undertake a second series of biosensor experiments in which GST::haPrP^{c} was mixed with 10 μM copper (II) before injection onto heparin (Fig. 2a). This addition had three consequences: the absolute signal strength was reduced, the binding curve tended to plateau more quickly (i.e. tended towards equilibrium more rapidly) and bound protein was apparently resistant to removal with a strong salt solution. A physiological concentration of copper (II) at synaptic terminals (a tissue rich in PrP) is in the region 10-20 μM (Brown *et al*., 1997). Fig. 2b displays the responses generated on heparin by sequential injections of GST::haPrP^{c} mixed with increasing concentrations (0-50 μM) of copper (II). The curve shapes altered dramatically as the concentration of Cu(II) was increased. As in Fig. 2A the rising phase of the curves assumed a more flattened shape toward the end of the contact phase and the high salt washes had a progressively smaller effect on the proportion of bound protein released. Note that the transient elevation of RU at the start of the dissociative period (indicated by the arrows “D”) was encountered inconsistently in experiments with full-length GST::haPrP^{c} and is difficult to explain in terms of movement of the protein (which should show net dissociation during this phase). A difference in the interaction between the running buffer and the derivatised and coated surfaces in such experiments may account for this effect though buffer-only injections were essentially without signal. In parallel experiments GST::haPrP^{c} was applied to an HS-derivatised surface in the presence or absence of Cu(II). In this case Cu(II) had no observable effect on the extent of binding (data not shown).

An ELISA for heparin / HS binding by GST-tagged PrP^c proteins and peptides was developed to confirm the findings of biosensor analysis. Fig. 3a shows data obtained when GST::haPrP^c was titrated on two heparins (bovine lung and porcine intestinal heparin) and porcine mucosal HS. Note that copper (II) was not added in these experiments. The binding signals on heparin proved to be much stronger than those generated by HS, but in both cases was dose dependent. Fig. 3b displays the results of a related ELISA in which GST::huPrP^c (1μg/ml) was applied to porcine intestinal heparin-coated wells in the presence of increasing concentrations of four divalent cations: Cu(II), Zn(II), Ni(II) and Mn(II). Copper (II) has the most dramatic inhibitory effect on the binding (maximal inhibition between 2-4 μM Cu(II)), whilst manganese (II) is apparently without influence. An intermediate level of inhibition was achieved in the presence of zinc and nickel. The ability of copper (II) to disrupt binding of GST::PrP^c to heparin supports data from biosensor experiments and it is interesting that in both methodologies high levels (50μM) of copper (II) fails to abolish heparin binding by GST::PrP^c completely. A primary purpose of the ELISA was to grade a number of sulfated polysaccharides on their ability to disrupt the interaction between GST::haPrP^c and heparin. Fig. 3c, d shows the results of incubation of GST::haPrP^c on PIH coated wells in the presence of increasing concentrations of sulfated GAGs, in the presence or absence of Cu(II). Similar data were obtained for binding to immobilised BLH (data not shown) and BLH and bovine kidney HS (BKHS) produced similar inhibitory profiles to PIH and PMHS respectively (data not shown). Irrespective of the source of heparin used as capture reagent, pentosan polysulfate (PPS) and dextran sulfate (MW8000, DS8) were the most potent inhibitors of heparin binding (on a weight for weight basis). The heparins were substantially weaker by comparison and both the low molecular weight PI heparin (LMW PIH) and two sources of HS were without effect. When copper (II) was added, the inhibitory activity of the GAGs/ polysaccharides was generally potentiated. Intact PIH (and also BLH, data not shown) now approached DS and PPS in inhibitory effect and the binding of GST::haPrP to PIH was weakly (but incompletely) disrupted by LMW PIH. These findings support the conclusion that Cu(II) weakens the interaction between full-length hamster PrP and heparin.

In a separate experiment human recombinant PrP^c was allowed to bind to PI-heparin in the presence of a selection of GAGs and modified heparins, provided at 10 and 100 μg/ml (Fig. 4). Again BLH was a good inhibitor of heparin-binding, whereas PMHS had essentially no effect. Both persulfated (oversulfated) heparin and selectively de-6-*O*-sulfated heparin were less effective inhibitors than unmodified BLH. de-2-*O*-sulfated heparin lacked inhibitory activity completely at 10 μg/ml and inhibited only weakly at 100 μg/ml. These results indicate

an important role for 2-*O*-sulfate groups in the prion-heparin interaction. Interestingly, low concentrations of 2-*O*-desulfated heparin, like CS (see also Fig. 3c) appear to promote PrP^c binding to immobilised heparin.

Binding of GST::hamster PrP peptide fusions and synthetic PrP peptides (of human sequence) to immobilised heparin / HS

To identify regions of the PrP molecule with independent heparin / HS binding activity, recombinant GST::fusions of partial hamster PrP sequences which collectively spanned the entire hamster prion sequence (Hundt *et al*., 2001; Weiss *et al*., 1995) were injected over a heparin-derivatised biosensor surface. When several independent batches of peptides were tested (no more than 2 months after expression-purification), P2 (53-93) and P4n (110-128) consistently yielded the most significant binding to heparin (Fig. 5a). P1, P3, P4, P5 and Px gave weak biosensor responses in general, although significant binding of P1 and P5 to heparin was recorded in a few batches. Note that the increase in signal occurring with P4n injection was not reversed by high salt, weak acid, alkaline or soluble heparin washes: procedures which should have disrupted all but the strongest electrostatic interactions. Peptides were tested in succession (as in Fig. 5a) and individually with no evidence of sample order-related enhancement or reduction of signal strength for any peptide. In some experiments FGF-receptor (a well-characterised heparin-binding protein) was injected before and after a series of the PrP peptides and no change was observed in the extent of binding of this protein (data not shown).

Subsequent experiments revealed that the heparin-binding activity of both GST::P2 (53-93) and GST::P4n (110-128) were sensitive to copper (II) addition, elevating and suppressing biosensor response respectively. GST::haPrP P2 (53-93) produced the most significant response when applied to the HS-derivatised biosensor surface but in contrast to its binding of heparin, recognition of HS was not influenced by Cu(II) (data not shown).

Although GST itself displays no heparin-binding activity in the buffer conditions chosen for GST::haPrP/peptides (data not shown), it was conceivable that the GST portion of the fusion could affect heparin binding activities of the contiguous PrP sequence. Indeed such an effect may explain the inconsistent binding observed for some GST-linked peptides (particularly GST::P5 (218-231). For this reason synthetic PrP peptides with no tag were also tested for binding to bovine lung heparin. (Fig. 5b). As the biosensor instrument transduces mass changes at the detector surface, low molecular weight analytes such as peptides tend to produce relatively weak signals per mol of bound analyte. By working at high sample

concentrations (in the range 0.1-1.0 mg/ml) the signal strength produced by low molecular analytes can be maximised, so long as surface binding sites are non-limiting. However such data should be interpreted with caution as high analyte concentrations can produce rate-limiting diffusional artefacts, re-binding phenomena, and increase the tendency for self-self interactions. Of the synthetic peptides only P1 (23-52), P2 (53-93) and P4n (110-128) yielded positive biosensor responses indicating that these three sequences are the strongest candidate heparin/ HS-binding regions in the prion molecule. Again peptides were tested singly and in different order of injection with no effect on the patterns of binding.

All GST-fusion peptides were tested for heparin binding by ELISA. Only GST::P1 (23-52) bound heparin reproducibly, providing a signal that was selectively inhibited (Fig. 6a). Note that the heparin-binding exhibited by P1 (23-52) like full-length PrP, was not competed by chondroitin sulfate. In order to explore the potential for intermolecular PrP-PrP interactions via the peptide sequences under investigation, GST::haPrP^{c} was incubated in microtitre wells coated with synthetic peptides P1 (23-52), P2 (53-93) and P4n (110-128). No other peptides were screened. A clear interaction was observed between the full-length PrP and imobilised P1 (Fig. 6b) This binding was not significantly inhibited by addition of soluble heparin, pentosan polysulfate or dextran sulfate which suggests a predominantly hydrophobic basis.

Additional biosensor experiments indicated that synthetic peptides P1 (23-52), P2 (53-93) and P4n (110-128) were capable of binding HS (data not shown), however only P2 (53-93) bound both GAGs in a concentration dependent manner (Fig. 7a, b). Heparin recognition was successfully competed with soluble heparin (data not shown) and porcine intestinal HS, though a weak inhibitor of the binding of synthetic P2 to immobilised heparin (data not shown) was an effective competitor for the binding of P2 to an HS-derivatised surface (Fig. 7c). It was noted that P2 (53-93) bound to the HS-derivatised surface in the presence of soluble PMHS was more readily eluted with the wash sequence (BLH, NaCl, NaOH) than P2 bound in the absence of competitor.

Copper (II) ions substantially enhance the heparin binding activity of synthetic peptide P2 (53-93)

As P2 (53-93) encompasses a region of PrP containing motifs for copper binding, biosensor experiments were conducted to assess any influence of copper availability on the heparin binding activity of this peptide. The biosensor response produced by 1 mg/mL P2 (53-93) was substantially enhanced (~ 7-fold) by the addition of 10μM Cu(II) (Fig. 8a). Intriguingly a second injection of P2 peptide (without added copper) applied immediately after one

containing Cu(II) generated a higher signal (ca. 2-3 fold) than the initial injection of P2 over a washed heparin surface. This strongly suggested that a proportion of the enhancing activity of Cu(II) might be exercised in a complex with the heparin substrate. To investigate this further we compared the accumulation of synthetic P2 on the heparin surface after two pre-treatments: i.) after injection of Cu(II) alone and ii.) after injection of Cu(II) followed by EGTA (a potent chelator of copper) (Fig. 8b). Injection of a brief pulse of EGTA between injections of Cu(II) and P2 greatly reduced the enhancement possible by pre-treatment with Cu(II) alone. Demonstrating the selectivity of this effect no enhancement of response was recorded when Cu(II) was applied in advance of an injection of P1 peptide (Fig. 8b) or P4n peptide (data not shown). To assess the ability of alternative metals to enhance heparin-binding by P2 (53-93), P2 (53-93) the peptide was injected in the absence of additional cation and also in the presence of 50μM copper (II), magnesium (II), nickel (II) or manganese (II) (Fig. 8c). None of the alternative cations replicated precisely the action of copper. Neither magnesium (II) or manganese (II) affected binding significantly and although nickel (II) induced a larger absolute response the rapid return of signal to baseline at completion of the contact period was indicative of a very weak interaction. Since copper (II) had an inhibitory influence on binding of PrP to heparin in the range 0-2 μM (Fig. 3B), we decided to apply P2 peptide at a range of Cu(II) concentrations (Fig. 8d). We subsequently found that the greatest enhancing activity was afforded at relatively high concentrations (>10 μM). In the concentration range that inhibits full-length PrP binding to heparin, Cu(II) had no clear influence on the behaviour of synthetic P2 (53-93) peptide.

Competitive ELISA to determine the relevance of P1, P2 and P4n sequences in the binding of full-length GST::haPrP^c to heparin

To access which sequences, singly or in combination, might contact heparin within the fully folded recombinant GST::haPrP^c molecule a competition ELISA was developed in which GST::haPrP^c was incubated on heparin in the presence of synthetic peptides P1, P2 and P4n (Fig. 9). It was anticipated that sequences contributing to heparin-binding in native PrP would compete with PrP for heparin when presented as peptides in solution. In this experiment synthetic peptides were either pre-incubated on heparin in advance of GST::ha PrP^c or mixed directly with GST::haPrP^c. Experiments were also conducted both in the presence and absence of added Cu(II). With no added Cu(II), none of the peptides affected the binding of GST::haPrP to heparin (Fig. 9a) yet when copper (II) was supplemented at 50 μM (a change which predictably reduced the binding signal due to GST::haPrP^c, maximum absorbance of ~

0.4 ODU versus 0.7 ODU) co-incubation with synthetic peptide P2 (53-93) resulted in a significantly lower binding signal on both BLH and PIH substrates than GST::haPrP^{c} alone (Fig. 9b).

Discussion

Prion proteins are placed firmly in the heparin-binding category of proteins. Not only have direct interactions been demonstrated with PrP (Brimacombe *et al*., 1999; Caughey *et al*., 1994), GAGs have been shown to influence PrP^{sc} accumulation in both cell-culture and *in vitro* converting experiments (Caughey and Raymond, 1993; Gabizon *et al*., 1993; Shaked *et al*., 2001a; Supattapone *et al*., 1999; Wong *et al*., 2001) and to modulate PrP^{sc} propagation and disease onset in animal models for scrapie (Beringue *et al*., 2000; Ladogana *et al*., 1992). This study has addressed three areas of current interest: the structural features of GAGs that engender PrP-binding ability, the influence of metal ions on GAG-binding and the location of GAG binding domains within PrP.

By both biosensor and ELISA techniques we were able to show direct binding of PrP to heparin, thus confirming earlier work (Brimacombe *et al*., 1999; Caughey *et al*., 1994). We were also able to demonstrate a direct interaction between PrP and purified heparan sulfate. This complements previous studies in which PrP-HS binding was strongly implicated but not directly demonstrated (eg. (Hundt *et al*., 2001; Shaked *et al*., 2001a; Wong *et al*., 2001)). Two aspects of the interaction with heparin were of particular interest. Firstly a weakened interaction in the presence of copper (II) ions was detected in several experiments (eg. Figs. 2a and b, 3b-d, Fig. 9) and the direct ELISA (Fig. 3b) indicated that this inhibition was only partial ie that significant proportion of heparin affinity remains even in high concentrations of copper. Nickel (II) and zinc (II), but not manganese (II) ions were also inhibitory, though not as strongly as copper. A related effect was first described by Brimacombe *et al.*, who reported that recombinant PrP could be displaced from an experimental nickel biosensor surface by pulses of heparin (Brimacombe *et al*., 1999). PrP loaded with copper and manganese assume distinct conformations with differing levels of protease-resistance (Brown *et al*., 2000; Miura *et al*., 1999; Qin *et al*., 2000; Shaked *et al*., 2001b; Wong *et al*., 2000). Our data suggests that one of the first consequences of metal induced conformational changes may be an altered affinity for endogenous HSPGs. Similarly, bioactive HSPGs may influence the uptake of metal ions by PrP.

The specificity of the PrP-heparin interaction was also investigated. The interaction of PrP with heparin (as detected by the ELISA) could be disrupted by soluble heparin, HS and by other sulfated polysaccharides (Fig. 3c, d and Fig. 4) but was refractory to inhibition by CS, confirming earlier work by others (Caughey *et al*., 1994). This last observation on the non-activity of CS has a special significance as it suggests that presentation of sulfates is not in itself sufficient for prion-binding activity. A particularly interesting finding was the potent inhibitory activity of a fraction of dextran sulfate of average MW 8000 (DS8). On a weight / volume basis this preparation was found to be superior to heparin and at least as effective as pentosan polysulfate (PPS), a polyanion with well-documented anti-prion activities in tissue culture and animal models (Ladogana *et al*., 1992; Shyng *et al*., 1995). It would be of great interest to compare the inhibitory activity of DS8 with fractions of higher MW such as DS500 (average MW 500,000) which is a particularly effective inhibitor of PrP^{sc} propagation in cell culture (Caughey and Raymond, 1993) and a potent anti-prion agent in animal studies of prion disease (Beringue *et al*., 2000). Using this competition ELISA we also explored the contribution to prion binding of 2- and 6-*O* sulfate groupings, two of the three types of sulfate which elaborate heparin and HS (N-sulfates were not examined in this study). Removal of 2-*O* sulfates was found to significantly reduce the inhibitory activity of BLH in the competitive ELISA (Fig. 4), suggesting an important role in prion-ligation. FGF-2 and hepatocyte growth factor are other heparin-binding proteins with a particular requirement for 2-O sulfate (Ono *et al*., 1999).

The location of heparin-binding sites in the prion protein has not been determined conclusively. This study revealed three sites in the recombinant molecule with independent affinity for GAGs, namely stretches 23-52, 53-93 and 110-128. While the first sequence (23-52) has no previously reported affinity for GAGs, there is some evidence for GAG binding activity in the other two. The biosensor response generated by P2(53-93) on both heparin and HS surfaces was concentration-dependent and selectively inhibitible and so of the three sequences heparin-binding is least likely therefore to be an artefact of aggregation in solution. As this sequence encompasses a major copper-binding motif its heparin-binding characteristics were also examined with and without added Cu(II) ions. In contrast to the intact molecule, accumulation of P2 (53-93) on heparin-binding was clearly enhanced by Cu(II) addition. The enhancing effect was not dependent on co-mixing and could be produced simply by conditioning the heparin surface with copper in advance of P2 (53-93) application. Certainly it is possible to form complexes between glycosaminoglycans and copper (II) *in vitro* and this is the basis of a sensitive detection method for heparin (Toida *et al*., 1997). This

supports a model in which copper ions may associate directly with the P2 sequence, with P2 following transfer from the heparin surface, or as part of a complex of all three components. It is difficult to square the binding properties of the intact molecule and P2 (53-93). Either an enhancing influence of copper on heparin-binding via P2 is not manifest in the native protein, or if present, is masked by another copper-modulated heparin-binding activity elsewhere in the sequence. A candidate for such a site is P4n (110-128) and preliminary data, not presented here, indicates that heparin-binding of P4n is abrogated by Cu(II). As with the complete molecule alternative metals had divergent effects on the binding of P2 (53-93) to heparin and it may be significant that manganese afforded no enhancing effect. Given its strong heparin-binding behaviour in biosensor studies, it was surprising that GST::P2 (53-93) was not identified as heparin-binding in the direct ELISA and the reason for this is not clear. Nevertheless in the presence of Cu(II) ions synthetic P2 (53-93) appeared to reduce heparin binding by co-mixed GST::haPrP^{c}. This result again supports a heparin-binding function for P2 (53-93) and suggests that the sequence is the predominant site for heparin recognition in the full-length protein

P1(23-52) demands particular attention as it proved positive in all tests of direct heparin and HS binding, including in the ELISA where concentration-dependence and target specificity were established (Fig. 6a). However as we were not able to demonstrate competition between this peptide and full-length GST::haPrP^{c} for binding to heparin (Fig. 9a, b) we propose that this sequence is a major heparin-binding site in intact PrP^{c}. A more important bindign partner may be sequences within PrP itself for we were able to show a direct interaction between the immobilised peptide and GST::haPrP^{c} (Fig. 6a). Such an interaction may be a means by which putative receptors for this sequence such as nucleic acids (Weiss *et al*., 1997) and Hsp60-like chaperonins could influence fibril formation.

In biosensor analysis P4n (110-128) bound heparin and HS both as a GST::fusion and as a free peptide, but presented no heparin binding in the ELISA. This peptide particularly yielded biosensor signals that could not be reversed by salt washing or extremes of pH. A possible explanation for this unusual behaviour is extensive aggregate formation either in solution or *in situ* at the heparin surface especially as a related sequence (106-126) shows a well described propensity for fibril formation (De Gioia *et al*., 1994; Gasset *et al*., 1992). Significantly the β-sheeted structures characteristic of peptide 106-126 in weakly acidic ionic buffers (conditions in which fibrils are also favoured) are evidently highly stable (resistant to 5% SDS or alkali to pH 12) (De Gioia *et al*., 1994). That P4n should show an affinity for heparin/HS was of interest as it has shown elsewhere that the cytotoxic properties of peptide

106-126 may be abrogated by addition of GAGs (Perez *et al*., 1998). Again however, an excess of P4n (110-128) did not interfere with the binding of full-length PrP to heparin and for this reason is considered a weaker candidate for a true physiological role in heparin/ HS binding than P2 (53-93).

To summarize we have identified three sites of heparin / HS binding activity in recombinant prion, and have shown that for at least one of these peptides designated P2 (residues 53-93) this activity is copper (II) sensitive. Which sequence makes the most important contribution to the interaction between full-length PrP and heparin is not clear though synthetic P2 peptide was shown to compete with the full-length prion molecule for binding to heparin. A prominent role for P2 (53-93) in direct HS binding was predicted from recent work demonstrating the strictly HSPG dependent binding of this peptide manner to mammalian cells (20). The behaviour of the sequence with native PrP must be different to that of the free peptide because of the significantly divergent effects of copper (II) ions on the two species. Lastly, the sensitivity of PrP^{c}-heparin binding to disruption with dextran sulphate and pentosan polysulfate, both of which have been proposed as candidate prophylactic molecules and the demonstration of intimate role of 2-*O* sulfate in heparin recognition was particularly interesting. Our data suggests that further investigation of the identified heparin-binding domains, and the potential specificity of carbohydrate binding sites in heparan sulfate, will lead to further insights into the role of HS in the function of prion proteins.

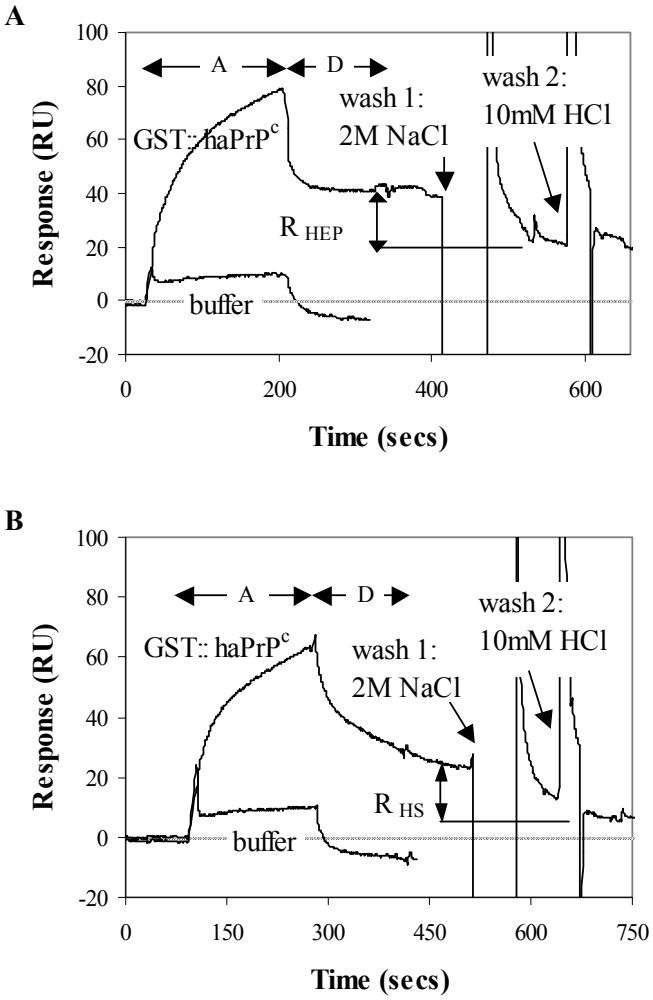

Fig. 1. Biosensor analysis of binding of full-length GST::haPrP^c to heparin and HS-derivatised surfaces. Sensorgrams produced by injection of recombinant GST::haPrP^c (30 µl, 15 µg/ml, 10µl/min flow rate) onto bovine lung heparin (A) and porcine intestinal heparan sulfate (B) coated biosensor surfaces (immobilised GAGs biotinylated mid-chain according to (Nadkarni and Linhardt, 1997)). The sensorgram displays the net response (in RU) for the the GAG-derivatised surface after subtraction of non-specific events as recorded on the non-derivatised control surface. The response changes recorded after injection of buffer alone have been superimposed ("buffer"). After a short period during which the sample makes contact with the control and heparin-derivatised surfaces (period A, 180 secs) the sample was exchanged for running buffer (period D, 120 secs) during which the the major movement of GST::haPrP^c was dissociation from the chip surface. After each injection of GST::haPrP^c the chip surfaces were washed with 2M NaCl and 10mM HCl. The amount of residual GST::haPrP^c removed from the heparin and HS surfaces by these washes is marked R_{HEP} and R_{HS} .

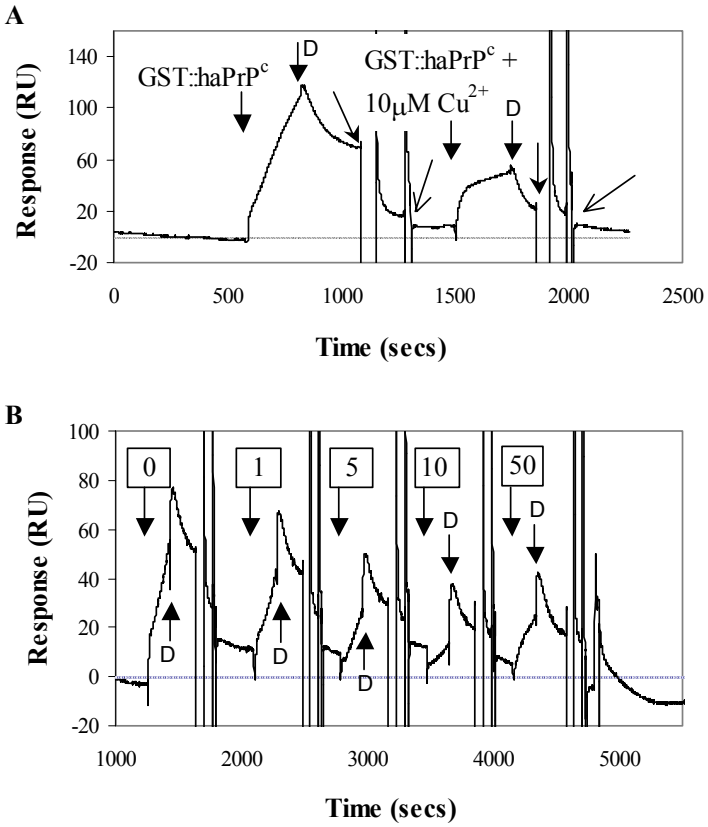

Fig. 2. Addition of Cu(II) ions suppress the biosensor response generated when GST::haPrP^c is injected onto heparin. (A) GST::haPrP (40 µl, 16 µg/ml) was injected over heparin (bovine lung, biotinylated at reducing termini, according to Nadkarni (Nadkarni and Linhardt, 1997)) in the absence of added Cu(II) ions, and subsequently in the presence of 10µM Cu(II). Initiation of dissociation phase for each sample application marked with letter D. (B) GST::haPrP^c (30 µl, 10 µg/ml) mixed with successively higher concentrations of Cu(II) (final concentration in µM shown in boxes) was injected over heparin. Initiation of injections marked by black arrows. Initiation of dissociation phase for each sample indicated by arrow with adjacent letter D. Between injections the chip surface was washed with 2 M NaCl (solid arrows) and 10mM HCl (open arrows). Note that neither buffer alone nor buffer containing 50 µM Cu(II) yielded a biosensor response on the heparin surface (data not shown).

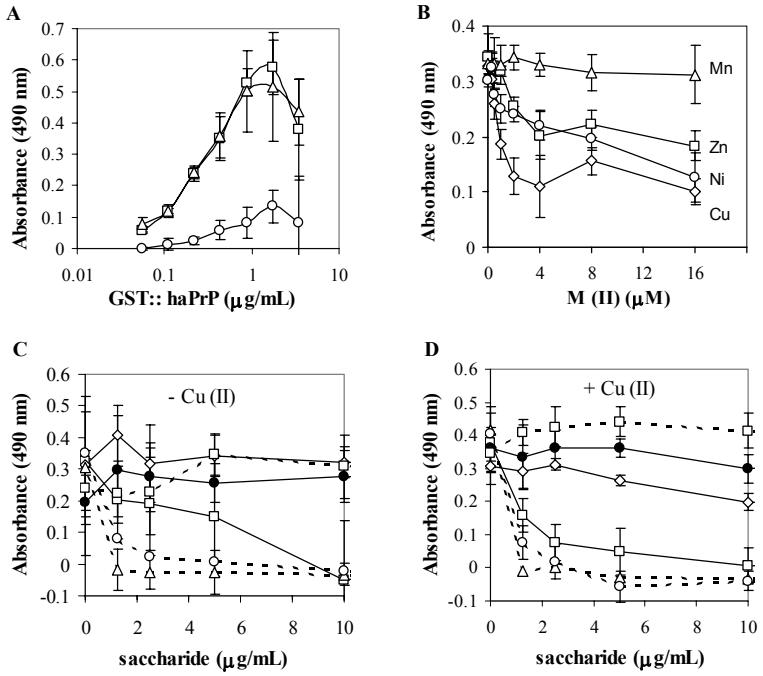

Fig. 3. ELISA analysis of PrP^c binding to heparin/ HS. Concentration and divalent cation dependency and inhibition with soluble GAGs. (A) ELISA signals generated by the application of increasing concentrations of GST::haPrP^c to immobilised BLH (triangles), PIH (boxes) or PMHS (circles). (B) Absorbance signals generated by incubation of 1μg/ml GST::huPrP^c onto PIH coated wells in the presence of increasing concentrations (0-16μM) of Mn (II), Zn (II), Ni (II) and Cu(II). (C, D) ELISA experiments in which GST::haPrP^c was incubated on immobilised PIH in the presence of increasing concentrations of sulfated polysaccharides and GAGs. The experiment was conducted in the absence (B) or presence (C) of 50 μM Cu(II). Inhibitors are as follows: PIH: solid line, open square; LMW PIH: solid line, open diamond; PMHS: solid line, closed circle; PPS: dashed line, open triangle; DS8: dashed line, open circle; CS: dashed line, open square. In all ELISA experiments values represent mean ± standard deviation of net absorbances (background subtracted) achieved from three experiments.

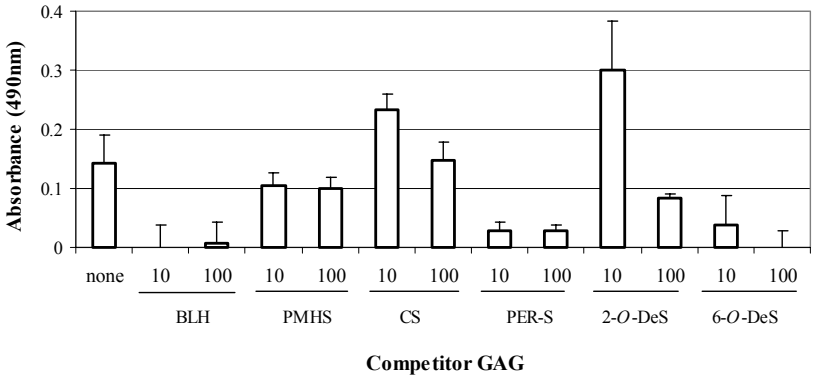

Fig. 4. Contribution of 2-*O* and 6-*O* sulfation to heparin recognition by PrP^c. Data from ELISA in which GST::huPrP^c (2.5 μg/ml) was incubated in wells coated with PIH with no addition or in the presence of selected GAGs (BLH, PMHS, CS) and chemically modified heparins at 10 and 100 μg/ml. PER-S: oversulfated bovine lung heparin; 2-*O*-DeS: de-2-*O*-sulfated BLH; 6-*O*-DeS: de-6-*O*-sulfated BLH.

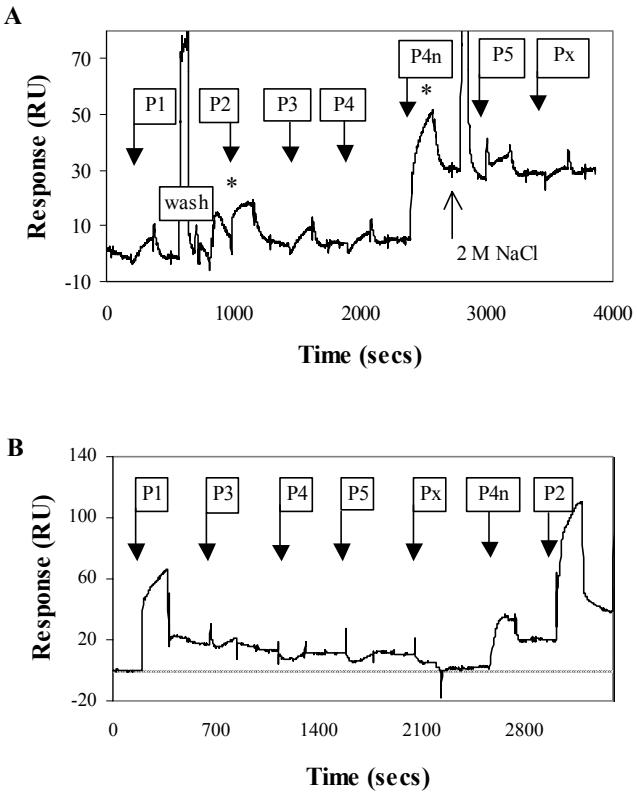

Fig. 5. Biosensor analysis of heparin-binding by GST-tagged and synthetic peptides. (A) GST::haPrP peptides were applied to a heparin surface (BLH modified with biotin at mid-chain sites according to (Nadkarni and Linhardt, 1997)) at the following concentrations (μg/ml): P1(23-52), 50; P2 (53-93), 50; P3 (90-109), 100; P4 (129-175), 80; P4n (110-128), 110; P5 (218-231), 80; Px (180-210), 120. Injection volumes were 30 μl. Asterisks mark the sample applications (GST::P2 and GST::P4n) that yielded significant biosensor responses. The surfaces were washed with 10 μl 2 M NaCl, 5 μl 10 mM HCl and 5 μl 10 mM NaOH following the injection of GST::P1 and washed with with 10 μl 2 M NaCl following injection of GST::P4n. (B) Synthetic peptides P1 (23-52), P2 (53-93), P3 (90-109), P4 (129-175), P4n (110-128), Px (180-210) and P5 (218-231) of human sequence were applied at a concentration of 1.0 mg/ml (volume 30μl) to a bovine lung heparin-derivatised surface (mid-chain biotinylated).

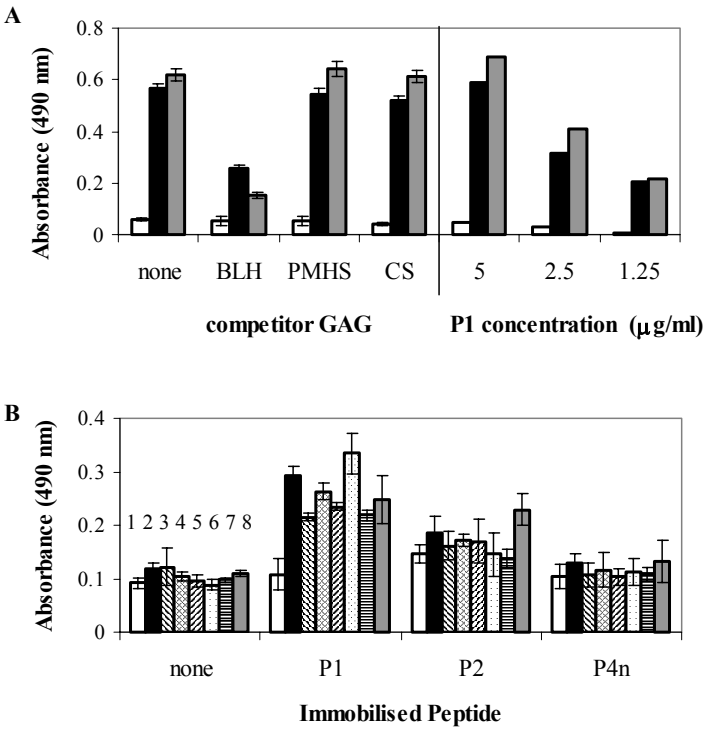

Fig. 6. Heparin binding of P1 (23-52) is inhibited by soluble heparin but not PMHS or CS and immobilised P1 peptide, but not P2 or P4n binds PrP^c. (A) This histogram shows the results of an ELISA analysis to determine the inhibitory effect of soluble GAGs, bovine lung heparin (BLH), chondroitin sulfate (CS) and porcine mucosa heparan sulfate (PMHS) all at 20 μg/ml, on the binding of GST::P1 (23-52) (5 μg/ml) to immobilised heparins. Target surfaces are as follows: un-modified control surface (white column); BLH (black column); PIH (shaded column). The right-hand side of the histogram depicts the absorbances achieved when a dilution series of GST::P1 (23-52) is applied to the same surfaces in the absence of competitor GAGs (single measurements, no error bars). (B) Histogram showing the signals produced in an ELISA to detect binding of GST::$haPrP^c$ to immobilised peptides P1, P2 and P4n. The following GAGs were co-incubated with GST::$haPrP^c$ at 100 μg/ml to assess the GAG-dependency of any interaction: column 1: buffer only (no $haPrP^c$, no GAG); column 2: GST: $haPrP^c$ only (no GAG), column 3-8: $haPrP^c$ + BLH, PMHS, PPS, DS, CS and LMW PIH respectively.

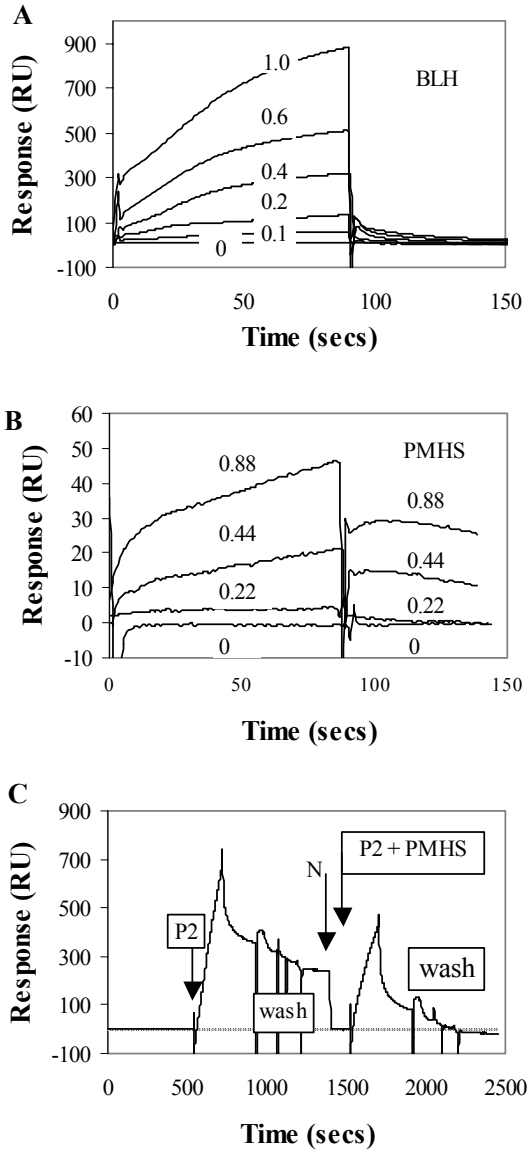

Fig. 7. Biosensor response due to binding of synthetic P2 (53-93) peptide to heparin and HS is concentration dependent and HS recognition is sensitive to competition with soluble HS. Synthetic P2 (53-93) was applied to BLH- (A) and PMHS-derivatised (B) surfaces at a range of concentrations (values in mg/ml indicated above the respective sensorgram profiles, overlaid on a common axis). (C): injection of 1 mg/ml synthetic P2 (53-93) over an HS surface (HS biotinylated mid-chain according to Rahmoune method (Rahmoune *et al*., 1998)) in the absence (injection at t = 500 s) or presence of 20 μg/ml soluble PMHS (injection at t = 1500 s). At position (N) the baseline response on HS- and control channels has been artificially equated to facilitate comparison of the two binding curves. After each sample injection surfaces were washed with 10 μl BLH (2 mg/ml), 10 μl 2 M NaCl and 5 μl 10 mM NaOH.

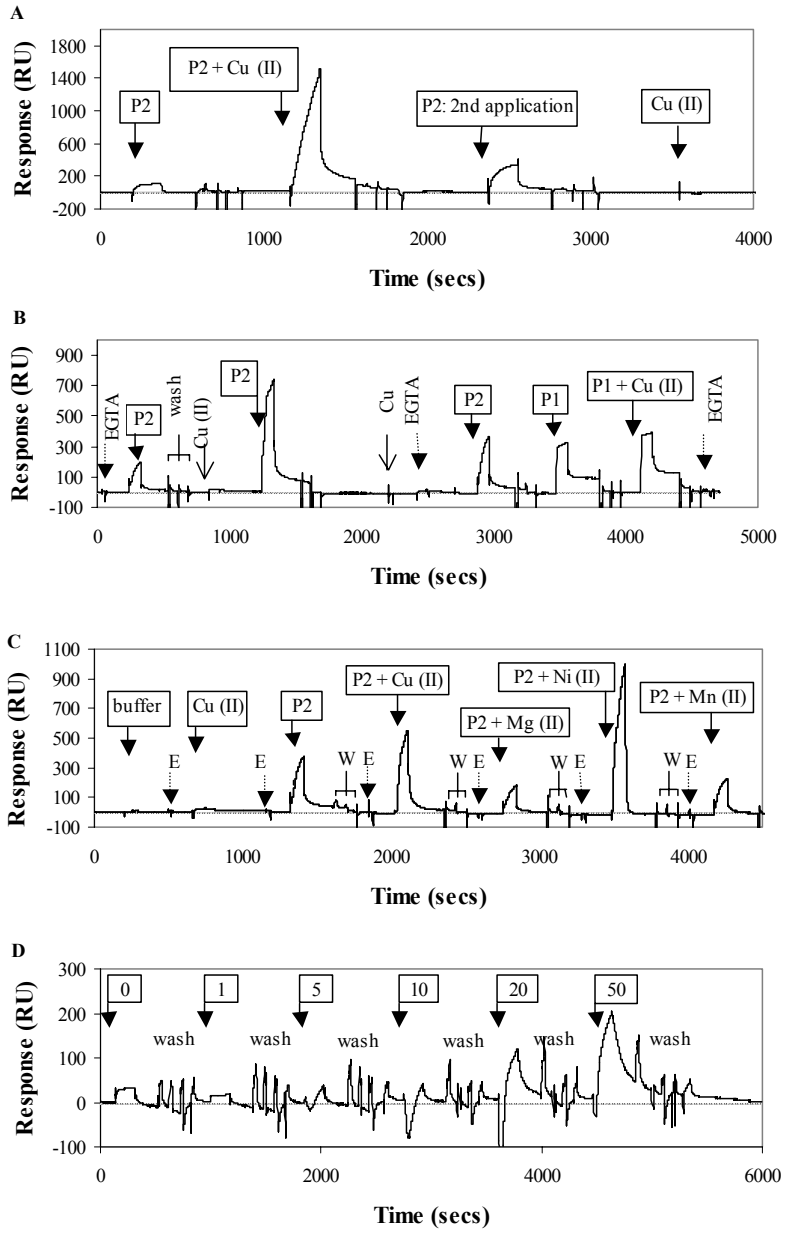

Fig. 8. The influence of Cu(II) on the biosensor response generated upon injection of P2 (53-93) over heparin. (A) Synthetic peptide P2 (53-93) was applied to a heparin surface at 1.0 mg/ml in HBS-P buffer (1st injection) or in buffer containing 10 μM Cu(II) ions (2nd injection). A third injection of peptide (2nd Cu-free application) bound more strongly than the first. This suggested that a sustained modification of the surface by the earlier (Cu(II) supplemented) injection had occurred. 10 μM Cu(II) alone (in the same buffer, injection # 4) produces no resonance change. (B) Synthetic P2 (53-93) peptide (0.25 mg/ml) was applied to the heparin surface following a pulse (10 μl, 2 mM) of EGTA (1st injection); a pulse (30 μl, 50 μM) of Cu(II) (2nd injection); a pulse of Cu(II) followed by a pulse of EGTA (3rd injection). Closed arrows (other than P2 applications) indicate applications of EGTA. Open arrows indicate applications of Cu(II). A triple wash regime of NaCl (2M), HCl (10mM) and NaOH (10mM) was applied immediately after each P2 injection. Beyond t= 3000s: synthetic P1 (23-52) peptide (0.25 mg/ml) applied alone or supplemented with 50 μM Cu(II). (C) Synthetic P2 (53-93) peptide (0.25 mg/ml) was injected with no added divalent cation, or mixed with 50 μM of each of Cu(II), Mg(II), Ni(II) or Mn(II) ions (total injected volume 30 μl). After each application of peptide the heparin (and control) surfaces were washed (W)

with NaCl (2M), HCl (10mM) and NaOH (10mM) and also with EGTA (2 mM, 10 µl) (arrows marked E). Injections of buffer alone and 50µM Cu(II) are indicated by the first two arrowed boxes and provide no response. (D) Sensorgram displaying biosensor responses on a BLH surface due to applications of synthetic P2 (53-93) peptide (33 µg/mL in HBS) mixed with increasing concentrations of Cu(II) (final Cu(II) concentration in µM shown in boxes). Between injections the heparin surface was washed with 5µL each of 2M NaCl, 10mM HCl, 10mM NaOH and 5mM EGTA.

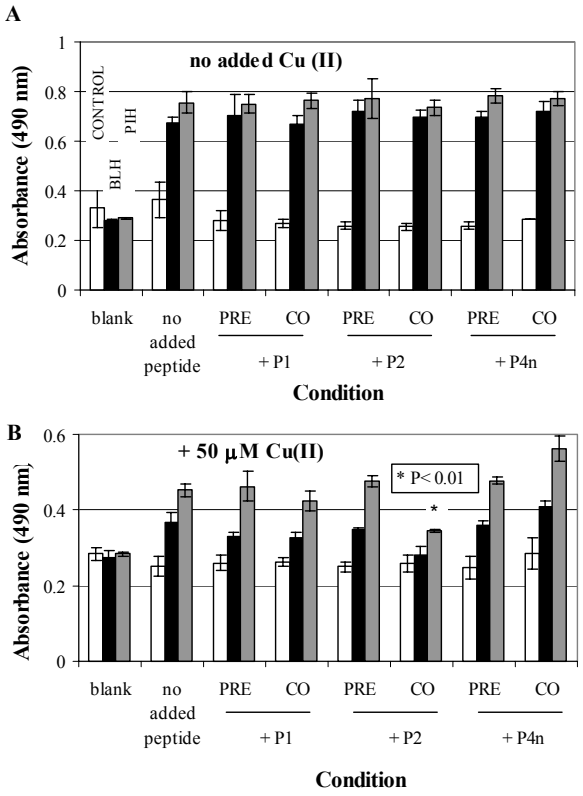

Fig. 9. Synthetic P2 (53-93) peptide interferes with the binding of GST::haPrP^c to immobilised heparin. (A) Histogram depicting the results of a two-phase ELISA designed to test the effect of pre-application or co-incubation of synthetic peptides P1, P2 and P4n on the binding of GST::haPrP^c to heparin. In the first phase each of the synthetic peptides (at 50 µg/ml) or buffer alone were incubated in wells of a microtitre plate pre-coated with heparin. In the second phase two combinations were tested: 1. GST::haPrP^c (1 µg/ml) or buffer alone was added to each of the wells (columns marked "PRE"). 2. a mixture of GST::haPrP^c (at 1 µg/ml) and each of the synthetic peptides (50 µg/ml) was added to wells pre-incubated with buffer only (columns marked "CO"). (B) The experiment was repeated in the presence of 50µM Cu(II) ions. Target substrates were as follows: un-modified (white column), BLH (black column) and PIH (shaded column). All procedures were replicated in wells lacking immobilised heparin (background controls). Error bars indicate the standard deviations of the net (background subtracted) absorbances from three wells. Control wells receiving only buffer (no GST::haPrP^c) presented a minimal background signal. Note that control wells which incubated with buffer alone or synthetic peptides followed by buffer (no GST::haPrP^c) generated a negligible signal (data not shown). The asterisk marks the data for GST::haPrP^c co-incubated with P2 (53-93) peptide. A students t-test comparison was applied to absorbance values generated by co-incubation on PIH of GST::haPrP^c with P2 (53-93) (data marked by asterisk) and incubation of GST::haPrP^c (no added peptide). The resulting probability value $P<0.01$ indicates that the reduction in ELISA signal as a result of co-incubation with P2 (53-93) is statistically significant.

Chapter VII

The prion protein-like Dpl shows a different interaction behaviour compared to PrP

submitted for publication:
Christoph Hundt and Stefan Weiss
The prion protein-like Dpl shows a different interaction behaviour compared to PrP
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Abstract

Recently, the prion-like protein termed Doppel (Dpl) was identified (Moore *et al*., 1999). This protein showed ~25% sequence identity with all known prion proteins. We have evidence that the cellular prion protein (PrP) is dimeric under native conditions (Hundt *et al*., submitted), a finding which was recently confirmed by the resolution of its crystal structure (Knaus *et al*., 2001). Human PrP interacts with its 37-kDa/67-kDa laminin receptor (Gauczynski *et al*., 2001b). The prion-like Dpl showed neither an interaction with itself nor with the laminin receptor in the yeast two-hybrid system. In addition, there is no interaction of the doppel protein with the cellular prion protein indicating that Dpl and PrP may be not tightly related.

Introduction

Prion diseases are fatal neurodegenerative disorders of mammals (Aguzzi and Weissmann, 1998; Prusiner, 1998; Lasmézas and Weiss, 2000). A major feature of these diseases is the conversion of the non-pathogenic, cellular prion protein PrP^{c} into its pathogenic isoform PrP^{Sc} (for review see (Prusiner, 2001)). This isoform has a strong tendency to polymerize forming amyloid aggregates. Recent publications demonstrate that PrP^{c} shows also dimerization properties. The crystal structure of human PrP reveals the dimeric nature of the prion protein (Knaus *et al*., 2001). We could also prove by gelfiltration that PrP is dimeric under native conditions and interacts with itself in the yeast two-hybrid system (Hundt *et al*., submitted). Besides the self-interaction of the prion protein the cellular receptor termed 37-kDa/67-kDa laminin receptor for the prion protein was identified which reveals direct and indirect interaction domains with PrP^{c} (Gauczynski *et al*., 2001b), (Hundt *et al*., 2001).

Recently, a prion-like protein termed Doppel (Dpl) was identified (Moore *et al*., 1999). The homology of Dpl and PrP is about 25% and the Dpl protein has a series of common features with cellular PrP (for summary see table 1). Although both proteins have different size they are processed during maturation and a signal peptide and a signal sequence was detached. Both proteins are glycosylated at two residues located at the C-terminal part of the proteins and share the same structural pattern: three α-helices and two β-sheets. Within the *Prn*-p-gene there are defined mutations/polymorphisms which affect transmissible spongiform encephalopathies (TSEs) in human and animals. Analogous there were some mutations investigated within the *Prn*-d-gene, but their relevance for the development of TSEs is not yet

proven. Besides this common features there are several differences between Dpl and PrP. One major difference represents the lack of the octarepeat region in the doppel protein located within the unstructured N-terminus of the prion protein. The region is responsible for copper binding (Stockel *et al*., 1998) and interacts with heparan sulfate proteoglycans (HSPGs) at the cell surface (Hundt *et al*., 2001) mediating the interaction of PrP with its 37-kDa/67-kDa laminin receptor via indirect interaction domains. These results indicate that the octarepeat region might be important for the physiological function of the prion protein. Since Dpl lacks the octarepeat region we hypothesize that Dpl cannot full-fill all functions of PrP. The proposed transmembrane region of PrP (aa 106-126) which seems to have neurotoxic effects (Haik *et al*., 2000) is also lacking in Dpl. In contrast to the prion protein, which exhibits one disulfide bridge due to two cystein residues encoded by the *Prn*-p-gene, the *Prn*-d-locus encodes four cystein residues, responsible for the formation of two disulfide bridges.

Materials, methods and results

In this manuscript we investigated whether Dpl reveals the same properties as PrP^c regarding self-interaction and binding to the 37-kDa/67-kDa laminin receptor employing the yeast two-hybrid system (for review see (Vidal and Legrain, 1999)). We PCR-amplified on cDNA level the mature form of Doppel termed Dpl27-154 employing oligodeoxy ribonucleotides flanking the Dpl-sequence introducing an *Eco*RI (5‘) and a *Sal*I (3‘) restriction site from cDNA generated from cultivated HeLa cells by RT-PCR. The PCR-product was cloned into the vector pSH2-1 via *Eco*RI and *Sal*I restriction sites resulting in pSH2-1-Dpl27-154. The Dpl27-154 encoding cDNA was excised from pSH2-1-via *Eco*RI (5‘) and *Sal*I (3‘) and subcloned into pJG4-5 restricted with *Eco*RI and *Xho*I resulting in pJG4-5-Dpl27-154. All plasmids were confirmed by didesoxy sequencing. The construction of pSH2-1-GST, pSH-2-1-GST::huPrP23-230 and pJG4-5-LRP44-295 was described previously (Rieger *et al*., 1997). The different bait and prey plasmids and the reporter plasmid pSH18-34 (*lacZ*) were co-transformed into EGY48 yeast cells and transformants were tested employing the β-galactosidase assay.

The Dpl27-154 protein failed to interact with GST::huPrP23-230 (Fig. 1 row 2). GST in prey position did not interact with Dpl (Fig. 1 row 1) demonstrating that there is no unspecific effect of the GST part of the GST::huPrP23-230 fusion protein. In contrast to GST::huPrP23-230 (Fig.1 row5) Dpl27-154 failed to interact with LRP44-295, the cellular receptor for PrP

(Fig. 1 row 3). In contrast to PrP which interact with each other (Hundt *et al*., submitted), Dpl27-154 did not interact with itself in the yeast two-hybrid system (Fig.1 row 4). To test whether Dpl 27-154 is expressed in the yeast two-hybrid system we co-transformed yeast cells EGY48 with pSH2-1-Dpl27-154 (bait-protein) and pJG4-5-Dpl27-154 (prey protein). Western-blot analysis with a polyclonal anti-Dpl antibody (kindly provided by H. M. Schätzl, Munich) revealed that the two fusion proteins were properly expressed (Fig. 2). The observed molecular weights of 21 kDa and 36 kDa for the bait and prey proteins match very well with calculated molecular weights of 21 kDa (lexA-DNA-binding domain: 7 kDa and Dpl27-154: 14 kDa) and 36 kDa (acidic activation domain B42: 22 kDa and Dpl27-154: 14 kDa). Therefore we can exclude that the lack of interaction of Dpl with PrP, LRP and itself is a consequence of non-expressed bait- and prey proteins.

Discussion

The incapability of Dpl to dimerize with itself was recently proposed by modelling the potential interaction interface (Warwicker, 2000). According to the model PrP dimerization might take place via a β-hairpin structure located between aa 119 and 128, a non-polar region lacking in the Dpl protein. We have further evidence that the major interaction domain for the PrP/PrP interaction is the octarepeat region (Hundt *et al*., submitted) which is highly conserved in different mammalian species (Schätzl *et al*., 1995). Besides the capability of the octarepeat region to bind HSPGs and therefore mediating the interaction between PrP and the 37-kDa/67-kDa laminin receptor (Hundt *et al*., 2001) one feature represents the binding of copper (Aronoff-Spencer *et al*., 2000) and the possibility to make a copper-mediated protein-protein interactions. In Alzheimer's disease, which represents a neurologic disease comparable to TSEs copper-induced aggregation is observed (Atwood *et al*., 1998). An analogous induction of the aggregation of the prion protein which occurs during the disease process might be conceivable. The octarepeat region is missing within the Dpl protein and therefore metal-induced dimerization is unlikely. In a yeast two-hybrid analysis we were able to detect a further interaction domain for the PrP/PrP interaction which is located in the core region of the prion protein between aa 90-230 (Hundt *et al*., submitted). This core region shows similarities to the structured region of Dpl which was analysed by NMR (Mo *et al*., 2001). Dimerization via this region is conceivable analogous to PrP. In contrast to PrP, Dpl is able to form two disulfide bridges confirmed by several methods (Mo *et al*., 2001). This may

confer the structure of Dpl a more rigid behaviour which probably prevent the attachment of a second Dpl molecule forming a dimer. The formation of two disulfide bridges in the Doppel protein might as well not allow domain swapping which contribute to dimer formation of PrP observed in the investigation of the crystal structure of PrP (Knaus *et al*., 2001).

The different structural features of PrP and Dpl might also be the reason for the absence of an interaction of Dpl with the 37-kDa/67-kDa laminin receptor, the receptor for cellular PrP (Gauczynski *et al*., 2001b). We can not exclude that additional factors which are not present in the nucleus of the yeast cells might mediate the Dpl-LRP interaction. For PrP HSPGs arbitrate the interaction with LRP/LR detected in cell binding assays (Hundt *et al*., 2001). Whether HSPGs or other factors have an influence on the binding of Doppel to LRP has to be further investigated.

Dpl fails to interact with PrP. This might be explained with the different expression patterns of PrP and Dpl *in vivo*. PrP is expressed mainly in neuronal tissues and in the brain, which is the most relevant location for the development of TSEs. In contrast, the main expression locus for Dpl represents the testis (Silverman *et al*., 2000) and the heart of wild-type mice (Moore *et al*., 1999). Dpl was not detectable in the brain of wild-type mice. Mice however, lacking the prion gene express Dpl in the brain (Moore *et al*., 1999). Therefore, the failure of interaction of PrP and Dpl in the yeast two-hybrid system is not astonishing. Whether Dpl can fulfill the functions of PrP in $PrP^{0/0}$ cells remains still unclear. The transport of copper and SOD activity proposed functions of the prion protein might not be adopted by Dpl due to the lack of the copper-binding octarepeat region. The role of Dpl in signal transduction necessary for cell survival postulated by Shmerling (Shmerling *et al*., 1998) has also to be further investigated.

Table 1: Comparison of Dpl and PrP

feature	**prion protein***	**doppel protein**	
universal properties:			
- protein size (human)	253 aa	179 aa	(Moore *et al*., 1999)
- mature form (human)	aa 23-230	aa 27-154	(Silverman *et al*., 2000)
- signal peptide / signal sequence	yes	yes	(Moore *et al*., 1999)
- disulfide bridge	1	2	(Moore *et al*., 1999)
- octarepeat region	yes	no	(Moore *et al*., 1999)
- copper binding	yes	no	(Moore *et al*., 1999)
- glycosylation sites	2	2	(Moore *et al*., 1999)
- mutation/polymorphism	at least 22 (human)	4 (human)	(Peoc'h *et al*., 2000)
- transmembrane region	aa 106-126 (supp.)	no	(Moore *et al*., 1999)
- structure	3 helices/2 β-sheets	3 helices/2 β-sheets	(Mo *et al*., 2001)
- pK treatment	sensitive (PrP^c)	sensitive	(Lu *et al*., 2000)
expression:			
- during RNA embryogenesis	yes	yes	(Moore *et al*., 1999)
- in the CNS	high level	low level	(Moore *et al*., 1999)
- $PrP^{0/0}$ mice	no	upregulation	(Moore *et al*., 1999)
- main local expression	brain	testis	(Silverman *et al*., 2000)
PrP^{Sc} propagation	PrP^c dependent	Dpl dispensable	(Behrens *et al*., 2001)
oxidative stress	SOD activity	induction of oxid. stress markers	(Wong *et al*., 2001)

* For citation see *Advances in Protein Chemistry, 2001 Vol. 57: Prion Proteins*, Academic Press

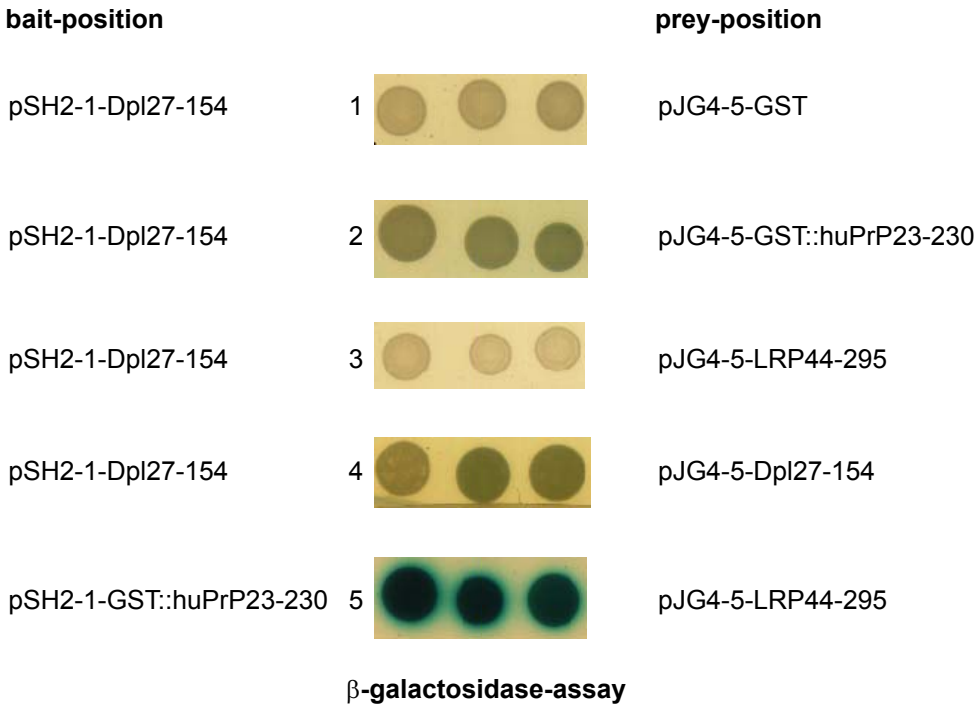

Fig. 1: Interaction study of the prion-like protein Dpl. HuDpl27-154 in bait-position (row 1-4) was co-expresseed with GST (row 1), GST::huPrP23-230 (row 2), LRP44-295 (row 3) and Dpl27-154 (row 4) in prey-position of the yeast two-hybrid system. For positive control GST::huPrP23-230 in bait position and LRP44-295 in prey position was co-expressed (row 5) (Hundt *et al*., 2001). Interactions were detected by the β-galactosidase reporter system.

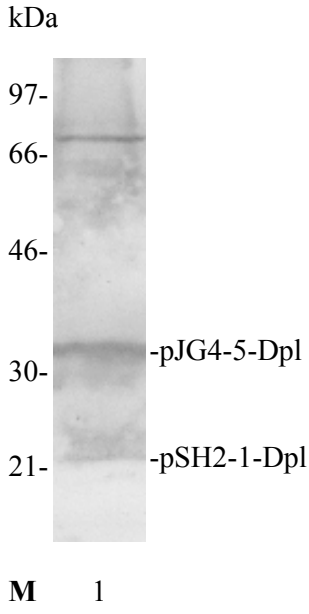

Fig. 2: Analysis of Dpl by SDS-PAGE and Western blotting. The expression of Dpl in bait and prey position of the yeast two-hybrid system was analysed on a 12,5% SDS-PA gel. Western blotting was performed employing a polyclonal Dpl-antibody (generous gift from H. Schätzl, Munich). Marker proteins are indicated.

Chapter VIII

Tyrosin kinase Fyn directly interacts with the cellular prion protein

prepared for publication:
Christoph Hundt and Stefan Weiss, 2002
Tyrosin kinase Fyn directly interacts with the cellular prion protein

Abstract

Recently the participation of the prion protein in signal transduction processes has been investigated (Mouillet-Richard *et al*., 2000). PrP mediated activation of the tyrosine kinase Fyn was shown opening new insights into the function of PrP. Here, we demonstrate the direct interaction of PrP with the tyrosine kinase Fyn employing the yeast two-hybrid system. In contrast, 37-kDa/67-kDa LRP/LR, the receptor for the prion protein, is not able to interact directly with tyrosine kinase Fyn. Since heparan sulfate proteoglycens (HSPGs) mediate the indirect interaction between 37-kDa/67-kDa LRP/LR and PrP, we propose a comparative model for the PrP mediated tyrosine kinase Fyn activation involving cell surface HSPGs.

Introduction

Prions have been extensively studied within the last twenty-five years and represent a new class of infectious agents causing transmissible spongiform encephalopathies (TSEs) (for review see (Prusiner *et al*., 1998; Lasmézas and Weiss, 2000; Aguzzi and Weissmann, 1998)). Many details are known about the biochemical features of the protein and the conversion reaction of the cellular prion protein PrP^{c} into its pathogenic isoform PrP^{Sc} (for review (Prusiner, 1998)). The physiological function of the prion protein, however, still remains unclear. Mice ablated from PrP are viable and develop quite normally (Bueler *et al*., 1992; Manson *et al*., 1994), others showed differences in circadian activity rhythms and sleep behaviour (Tobler *et al*., 1996) and altered synaptic functions (Collinge *et al*., 1994). The Purkinje cell degeneration observed in $PrP^{0/0}$ mice (Sakaguchi *et al*., 1996), however, was due to an overexpression of doppel, located downstream of the *Prn*-p gene (Weissmann, 1996). One major function of PrP is its necessity for the development of TSEs. Transgenic mice lacking the *Prn*-p gene were not able to propagate the disease after inoculation with the scrapie agent (Bueler *et al*., 1993). PrP might also be involved in the copper metabolism (Brown *et al*., 1997), since copper binds to the prion protein (Hornshaw *et al*., 1995). A SOD activity of PrP has been identified (Brown *et al*., 1999) indicating that PrP might play a role in the cellular resistance machinery against oxidative stress. Very recently, the role of PrP in signal transduction was investigated (Mouillet-Richard *et al*., 2000). The PrP^{c} dependent activation of the tyrosine kinase Fyn was demonstrated in neuronal cells. The activation might be mediated by caveolin or clathrin, which might trigger the signal from the exterior to the

interior of the cells. PrP^c anchores at the outer membrane, whereas tyrosine kinase Fyn locates at the inner plasma membrane. In contrast, there are some evidences that other factors mediate the signal transduction since PrP and Fyn are located in detergent resistant complexes without caveolin (Gorodinsky and Harris, 1995). Possible candidates are cadherins which interact with the Fyn kinase (Yagi and Takeichi, 2000) and represent also binding partners of PrP (Cashman and Dodelet, 1997).

One possible mediator for the signal transduction between PrP and the tyrosin kinase Fyn is the 37-kDa/67-kDa laminin receptor LRP/LR. This receptor, identified as an interactor for PrP^c (Rieger *et al.*, 1997), was characterized as the receptor for the cellular prion protein (Gauczynski *et al*., 2001b). LRP/LR is localized on the cell surface of mammalian cells spanning the cell membrane via a transmembrane domain stretching from aa 86 to aa 101 (for review see (Gauczynski *et al*., 2001a)). The C-terminus of LRP/LR is located to the extracellular space encompassing the laminin and PrP binding sites (Hundt *et al*., 2001). Therefore LRP/LR represents an suspicious candidate mediating the PrP signal transducion activity. The participation of PrP within the signal transduction cascades was enforced by the investigation of the susceptibility of the prion protein to enzymatic phosphorylation (Negro *et al*., 2000). Tyrosine kinase Fyn seems to mediate multiple signal transduction pathways via different molecules (Kai *et al*., 1997).

Material, methods and results

We now wanted to know whether the interaction of PrP with Fyn observed in the antibody-cross-linking experiment (Mouillet-Richard, 2000) is also detectable in the yeast two-hybrid system and therefore is direct in the absence of any co-factors. Recently, the yeast two-hybrid system identified besides an indirect HSPG mediated interaction between PrP and LRP/LR confirmed in cell binding assays (Hundt *et al*., 2001) a direct interaction between both molecules. This interaction was used as a positive control for PrP-Fyn interaction studies (Fig. 1 row 4).

To investigate the relationship between PrP, LRP and Fyn, we cloned the cDNA encoding human Fyn kinase into the bait plasmid of the yeast two-hybrid system by PCR using a cDNA encoding for human tyrosine kinase Fyn (kindly provided by A. Ullrich, Munich). The PCR product was cloned via *Eco*RI (5‘) and *Sal*I (3‘) restriction sites into the vector pSH2-1 resulting in pSH2-1-Fyn2-537. The preparation of the prey plasmids was described elsewhere

(Hundt *et al*., 2001). The different bait and prey plasmids and the reporter plasmid pSH18-34 (*lacZ*) were co-transformed into EGY48 yeast cells and transformants were tested employing the β-galactosidase assay.

Tyrosine kinase Fyn interacts directly with the human prion protein aa 23-230 in the yeast two-hybrid system (Fig.1 row 2). PrP was utilized as a fusion protein with GST. For specificity control no interaction between Fyn and GST was observed (Fig.1 row 1). In addition, no interaction between Fyn and LRP2-295 was detected (Fig.1 row 3) suggesting that either PrP dependent Fyn activation occurs directly or an additional factor may trigger the PrP dependent tyrosine kinase Fyn interaction.

Discussion

The prion protein is GPI-anchored on the surface of neuronal cells (Stahl *et al*., 1987). How can the extracellular prion protein interact directly with the intracellular tyrosine kinase Fyn? The expression patterns of PrP and Fyn in mammalian cells are similar. Both proteins are extensively expressed in neurons (Bare *et al*., 1993) and in the brain (Yagi *et al*., 1994; Caughey *et al*., 1988). PrP and Fyn are further detectable in detergent-resistant complexes lacking caveolin (Gorodinsky and Harris, 1995). Studies of PrP translocation at the endoplasmatic reticulum (ER) revealed new topologic forms of the prion protein (Hegde *et al*., 1998). Two transmembrane forms of PrP spanning the membrane of the ER in different orientations were identified termed ^{Ctm}PrP (C-terminal part of PrP is located to the lumen of the ER) and ^{Ntm}PrP (N-terminus located to the lumen of the ER). Mutations within the transmembrane region increased the level of the ^{Ctm}PrP form which was then detectable in the brain of transgenic mice (Hegde *et al*., 1998). Recently, the transmembrane forms were identified in *in vitro* translation systems (Stewart and Harris, 2001). The transport of transmembrane proteins processed in the ER and the Golgi complex is performed by vesicles. The transmembrane proteins were never released into the cytosol but transported to the plasma membrane via budding of the vesicle. PrP once stuck in the membrane of the ER might rest as a transmembrane protein at the membrane. Therefore, it is conceivable that transmembrane forms of PrP exist in wild-type cells in addition to the GPI-anchored PrP. These transmembrane forms might be able to interact with the intracellular tyrosine kinase Fyn. In normal cells, the signal transduction via these transmembrane forms does not seem to play a dominant role and the signal might be transmitted via other factors. In patients

suffering from GSS (Gerstmann-Sträussler-Scheinker syndrome) due to a mutation within the prion gene at position 117 (A117V) which favours the transmembrane form ^{Ctm}PrP, the direct signal transduction activity of PrP activating Fyn is probably increased. This enhanced signal transmission or the blockage of the Fyn kinase with ^{Ctm}PrP regarding regular functions of Fyn might perhaps lead to the progression of the disease. The effect of the mutation A117V on the interaction behaviour of PrP and Fyn has to be investigated in the yeast two-hybrid system. Recently, an interaction of PrP with the intracellular adaptor protein Grb2 involved in the intracellular signal transduction was identified employing the yeast two-hybrid system (Spielhaupter and Schatzl, 2001). Therefore direct interaction of PrP with molecules of the signal transduction cascade could be a common feature of the cellular prion protein.

The majority of the wild-type prion protein is located on the outer side of the membrane suggesting that additional factors might participate in signal transduction processes. One possible mediator might be the laminin receptor LRP/LR which binds to PrP in a direct and an indirect way (Hundt *et al*., 2001). The indirect interaction is mediated by heparan sulfate proteoglycans (HSPGs) located to the cell membrane. The interaction of HSPGs with other proteins is not detectable in the yeast two-hybrid system, due to the absence of HSPGs in the nucleus of yeast cells where the interaction occurs. There is some evidence that the tyrosine kinase Fyn is related to HSPGs. The proteoglycan N-syndecan (syndecan-3) was co-purified with Fyn from the hippocampus (Lauri *et al*., 1999) and both molecules were involved in neurite outgrowth (Kinnunen *et al.*, 1998). Therefore HSPGs could act as regulators transmitting signals between extracellular HSPG binding factors and the Fyn signalling pathway. This opens the possibility that during cell-cell-communication essential for cell survival, LRP interacts with PrP from one cell stimulating a signal cascade via HSPG to the cytosol of another cell (intercellular signalling) (Fig.2: I). Another possibility is intracellular signalling (Fig.2: II), whereas the signal is mediated by HSPGs from PrP to tyrosine kinase Fyn of the same cell. The cascades might lead to activation of Fyn and to further reactions within the cell. The involvement of other factors could not be excluded and has to be further investigated. The influence of HSPGs on the signal transduction and the consequences for the development of prion diseases comparable to Alzheimer‘s disease where HSPGs are accumulated in β-amyloid deposits (Snow *et al*., 1994) have to be further enlightened.

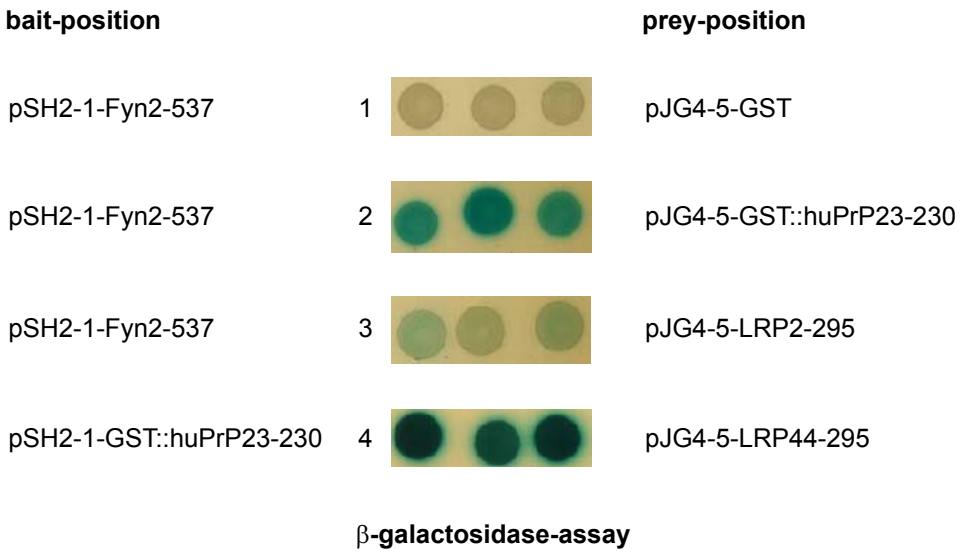

Fig. 1: Interaction study of the tyrosine kinase Fyn. Human tyrosine kinase Fyn in bait position (row 1-3) was co-expressed with GST (row 1), GST::huPrP23-230 (row 2) and LRP2-295 (row3) in prey position of the yeast two-hybrid system. For positive control GST::huPrP23-230 in bait position and LRP44-295 in prey position was co-expressed (row 4) (Hundt *et al*., 2001). Interactions were detected by the β-galactosidase reporter system.

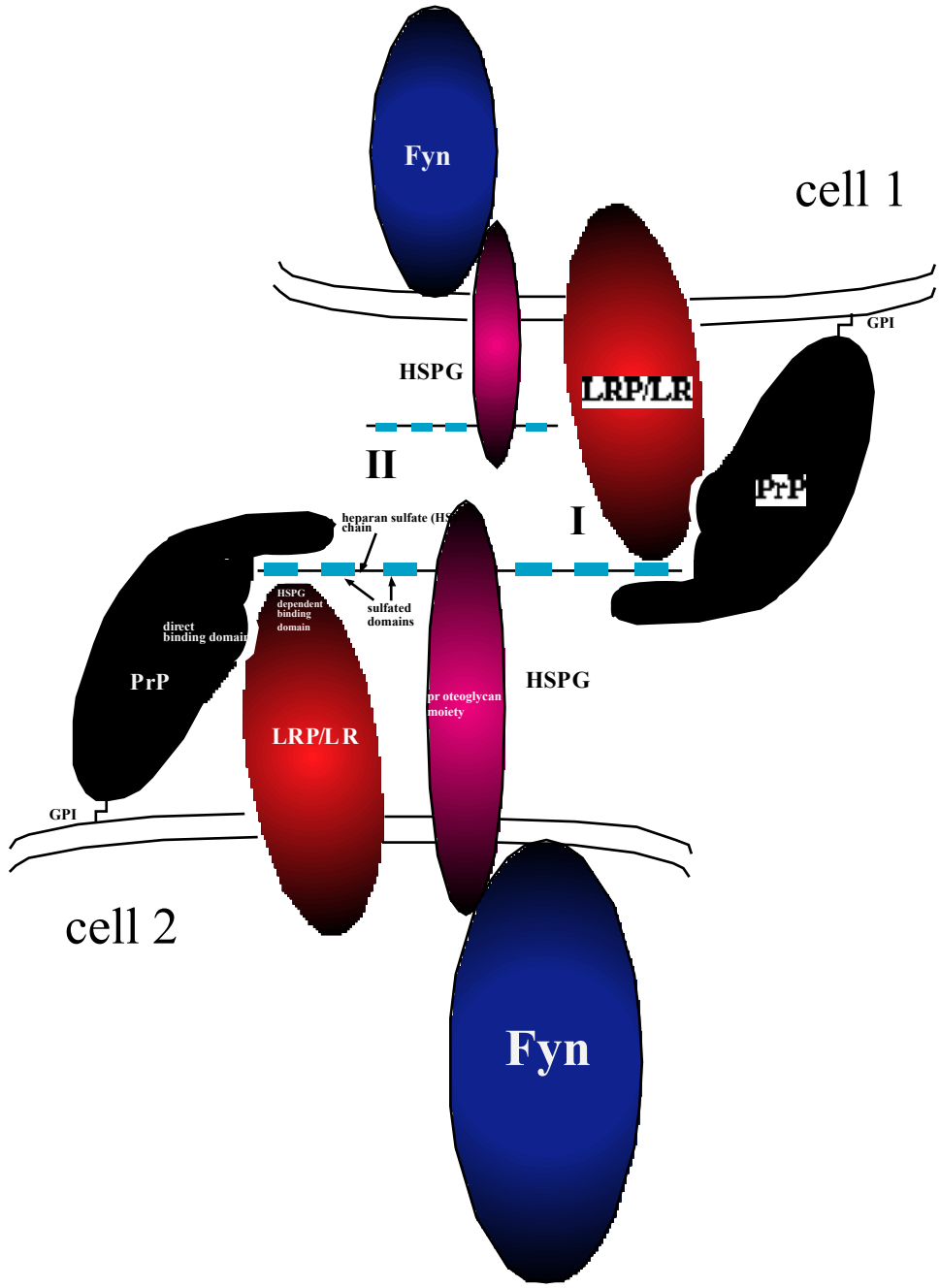

Fig. 2: Model for the signal transduction of the prion protein and the tyrosine kinase Fyn. PrP is located on the outer membrane of a cell and binds to its receptor LRP/LR via a direct and a HSPG-dependent binding domain (Hundt *et al*., 2001). The tyrosine kinase Fyn is located on the innerside of the cell and interact with the proteoglycan moiety of the HSPG. It is possible that the heparan sulfate arm of the HSPG molecule can mediate the binding of LRP/LR and PrP of another cell (intercellular signalling) (I) or of the same cell (intracellular signalling) (II). This will probably lead to the start of a signal cascade where Fyn is activated and phosphorylation of other factors might occur.

Chapter IX

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Abbreviations

aa	amino acid
Ab	antibody
AD	Alzheimer disease
AmB	amphotericin B
APP	amyloid precursor protein
ATP	adenosine triphosphate
BHK	baby hamster kidney
bovPrP	bovine PrP
bp	base pair
BSE	bovine spongiforme encephalopathy
CD	circular dichroism
cDNA	complementary DNA
CHO	chinese hamster ovary
CJD	Creutzfeldt-Jakob disease
CLDs	caveolae-like domains
CNS	central nervous system
CTP	cytosine triphosphate
CWD	chronic wasting disease
Cy2	carbocyanine
DAPI	4′-6-diamidine-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dpl	doppel
DTT	dithiothreitol
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylene-diamine-tetraacidic acid
EEG	electro encephalogram
ER	endoplasmic reticulum
FACscans	Fluorescence-activated cell scans
fCJD	familial CJD
FCS	fetal calf serum
FCS	fluorescent correlation spectroscopy
FFI	fatal familial insomnia
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
gal-3	galectin-3
GFAP	glial fibrillary acidic protein
GPI	glycosyl phosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
GST	glutathione *S*-transferase
h	hour
haPrP	hamster PrP
Hsp	heat shock protein
HSPG	heparansulfate-proteoglycan
huPrP	human PrP
IDX	4‘-iodo-4‘-deoxy-doxorubicin
IF	immunofluorescence
IgG	immunoglobulin G
kb	kilobases
k_D	dissociation konstant
kDa	kilo Dalton
LB	Luria Bertani medium
LBP	laminin binding protein
LR	laminin receptor
LRP	laminin receptor precursor
mAb	monoclonal Ab
min	minutes
moPrP	mouse PrP
mRNA	messenger RNA
MW	molecular weight
NMR	nuclear magnetic resonance
nvCJD	new variant CJD
OR	octarepeat
ORF	open reading frame
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPLC	phospholipase C
PK	proteinase K
Pli	PrP ligand
PMSF	phenylmethyl-sulfonylfluoride
PrP^c	cellular prion protein
PrPres	resistent prion protein
PrP^{Sc}	Scrapie (pathogenic)-isoform of PrP
PrPsen	sensitive prion protein
PVDF	polyvinyliden-difluoride
r.p.m.	rounds per minute
rec.	recombinant
rER	rough ER
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SAF	Scrapie associated fibrils
sCJD	sporadic CJD
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SFV	Semliki Forest virus
SOD	superoxide dismutase
TBS	tris buffered saline
TGN	trans-Golgi network
TMAO	trimethylamine-*N*-oxide
TMD	transmembrane domain
TSE	transmissible spongiforme encephalopathy
UK	United Kingdom
UTP	uracil-triphosphate
UV	ultraviolett
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YPD	yeast peptone dextrose medium