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Characterization of the 37-kDa/67-kDa laminin receptor as the cell surface receptor for the cellular prion protein

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Erklärung

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Ehrenwörtliche Versicherung

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Contents

Summary

Prions have been extensively studied since they represent a new class of infectious agents in which a protein, PrP^{Sc} (prion scrapie), appears to be the sole component of the infectious particle. They are responsible for transmissible spongiform encephalopathies (TSEs), which affect both, humans and animals. Human prion diseases occur in infectious, sporadic or genetic forms. The "protein only" hypothesis argues that the key event in the pathogenesis represents the conversion of the normal host protein, PrP^{c}, into its pathogenic isoform PrP^{Sc}. Prion diseases have been associated with the accumulation of this abnormally folded protein and its neurotoxic effects. However, it is not known if PrP^{c} loss of function is an important factor since the normal biological function of PrP^{c}, a cell surface-anchored glycoprotein predominantly expressed in neuronal cells, and the cellular processes in which this protein is involved remain obscure.

Recently, the human 37 kDa laminin receptor precursor (LRP), which represents the precursor of the human 67 kDa high-affinity laminin receptor (LR), was identified as a binding partner for the cellular prion protein in a yeast two-hybrid screen. In order to characterize the possible role of LRP/LR as a cell surface receptor for PrP^{c}, cell culture studies were performed to investigate the cellular localization of PrP and LRP/LR and to analyse the binding and internalization behaviour of PrP depending on the presence of LRP/LR on the cell surface of neuronal and non-neuronal cells. Immunofluorescence analysis of non-permeabilized murine neuroblastoma cells demonstrated that PrP and LRP/LR co-localize on the surface of these cells. In addition, baby hamster kidney (BHK) cells transfected with recombinant Semlik-Forest virus RNAs overexpressed human PrP and human LRP at their cell surface, the latter one orientated as a type II transmembrane protein with its C-terminus outside and its N-terminus inside the cell. Co-localization of both proteins was observed on BHK cells co-transfected with LRP and PrP encoding recombinant SFV RNAs. Cell binding and internalization assays with recombinant human PrP demonstrated the LRP/LR-dependent binding and endocytosis of externally added human PrP. An increased, dose-dependent cell binding of recombinant PrP was demonstrated by BHK cells overexpressing full-length human LRP on their cell surface. Trypsin treatment of the cell surface revealed the LRP dependent internalization of GST-tagged and

untagged, glycosylated PrP. In contrast to wild-type LRP, the expression of an LRP mutant lacking its transmembrane domain led to the secretion of this mutant from transfected BHK cells and totally abolished the binding and internalization of exogenous, recombinant PrP. This LRP mutant could function as a decoy recetor in therapy of TSEs. The strict LRP/LR specificity of the PrP binding to neuronal cells was verified by testing the displacement capacity of a series of different antibodies in the LRP-PrP binding reaction. Only LRP and PrP specific antibodies were able to block totally the binding of human GST-fused PrP to N2a and NT2 cells whereas various control antibodies used for competition showed no effect. Mapping analyses in the yeast two-hybrid system and cell-binding assays identified direct and heparan sulfate proteoglycan (HSPG)-dependent interaction sites mediating the binding of cellular PrP to the 37-kDa/67-kDa LRP/LR. The relationship between the 37-kDa LRP and the 67-kDa high-affinity LR is unknown so far. Both forms were observed in plasma membrane fractions of N2a cells. We conclude from these data that the 37-kDa/67-kDa laminin receptor acts as the main cell surface receptor for PrP.

High-level expression and purification of recombinant, glycosylated prion proteins in mammalian cells is essential for a better understanding of the physiological function of PrP^{c} and biochemical processes responsible for prion diseases. Due to the presence of important organelles, membranes and other cellular cofactors which are necessary for the correct processing, trafficking and localization of prion proteins mammalian cell culture systems such as the Semliki-Forest virus (SFV) system allow the synthesis and characterization of wild-type as well as mutant PrP to get a better insight into the biology of these proteins. Therefore, the SFV system was used to generate recombinant highly glycosylated human wild-type and human disease-associated mutant prion proteins as well as FLAG-tagged human and bovine PrP in cultured BHK cells. Both mutated variants, which are related to the human prion diseases fatal familial insomnia (FFI) and Creutzfeldt-Jakob disease (CJD) reveal proteinase K (PK) resistance, one of the most typical biochemical properties characteristic for the infectious scrapie isoform of the prion protein. The subcellular location of both PrP mutants at the cell surface and in intracellular compartments of transfected BHK cells was similar to that of wild-type PrP without any significant differences regarding the cellular distribution and expression level. In addition, FLAG-tagged prion proteins

were expressed with high efficiency in BHK cells showing the typical glycosylation pattern allowing the rapid and simple purification via anti-FLAG antibody chromatography.

PrP dimers could play an essential role in the $PrP^{c} \rightarrow PrP^{Sc}$ conversion process and might be involved in PrP interspecies transmission. Recently, crystallization of the prion protein in a dimeric form was reported. Size exclusion chromatography showed that native soluble homogeneous FLAG tagged prion proteins from hamster, man and cattle expressed in the baculovirus system were predominantly dimeric. The PrP/PrP interaction was confirmed in rec. 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.

The identification of the 37-kDa/67-kDa laminin receptor as the receptor for the cellular prion protein might represent an important step for a better understanding of the molecular biology of prion diseases and might lead to the development of powerful therapeutics such as LRP/LR specific antibodies for the treatment of these unconventional diseases.

CHAPTER I

Introduction

1. Prion diseases in humans and animals

Transmissible spongiform encephalopathies (TSE) represent a special form of fatal neurodegenerative diseases affecting humans and animals. Human TSEs include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Kuru. The most known prion diseases in animals are Scrapie in sheep, bovine spongiform encephalopathie (BSE) in cattle and chronic wasting disease (CWD) in elk and deer (for review see (Lasmézas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann and Aguzzi, 1997)). The causative agent of these disorders is termed prion which is defined as a proteinaceous infectious particle constituted of the abnormal pathogenic isoform, PrP^{Sc}, of the host-encoded cellular prion protein, PrP^{c} (for review see (Prusiner, 1998)). The alteration in protein conformation is thought to be the basic event for the generation of pathogenesis produced by a misfolded, oligomeric protein in the absence of any specific nucleic acids (Kellings *et al.*, 1992). Therefore, based on the "protein-only" hypothesis, prions represent a new class of infectious pathogens (for review see (Prusiner, 1991)).

1.1. History and Epidemiology

TSEs have a long and exciting history. Scrapie, the prototype of prion diseases in animals affecting sheep appeared for the first time in the United Kingdom (UK) and in Germany over 200 years ago (for review see (Prusiner, 1997)). The observation that the affected animals ″scrape″ on hedges in the early stage of the disease resulted in the term ″Scrapie″. During the progression of the disease these sheeps staggered, collapsed and finally died. The pathological examination of the brain revealed spongiform degeneration accompanied by amyloid-like plaques, astrocytosis and gliosis (for review see (Lasmézas and Weiss, 2000)).

In the 1950s Carlton Gajdusek, the later Nobelist, discovered aborigines in New Guinea suffering from a human form of TSE that resulted from tribal cannibalism and which was later termed Kuru (Gajdusek and Zigas, 1957). One typical characteristic of this degenerative disorder was the appearance of "kuru-type" plaques in the brain of affected persons. The prohibition of this kind of cannibalistic ritual act by law decreased dramatically the incidence rates of people suffering from Kuru.

In the 1920s the two German physicians Creutzfeldt and Jakob described brain lesions in humans similar to those observed in the brains from Kuru-affected aborigines such as spongiosis, astrocytosis and gliosis. This human prion disease was termed Creutzfeldt-Jakob disease (CJD) (Creutzfeldt, 1920) and occured with up to 85% sporadically and with up to 15% familial (Masters, 1981). Two further human TSEs have been identified which differ

from classical CJD (sporadic and familial cases) in their clinical and pathological criteria and are strictly genetically linked: the Gerstmann-Sträussler-Scheinker syndrome (GSS) (Gerstmann, 1928) and the fatal familial insomnia (FFI) (Tateishi *et al*., 1995).

The BSE epidemic and crisis started in the UK in 1985 when the first suspected cases of this disease were observed. In November 1987 the first case of BSE was histologically confirmed (Wells *et al*., 1987). In the following years the number of cases increased continuously and the crises raised its maximum in the late 1992 and in the beginning of 1993 with 3500 confirmed new infections each month (source: ministery of agriculture, fisheries and food (MAFF), UK). What was the reason for this epidemic affecting cattles in the UK? According to the sheep origin hypothesis, the causative pathogens were transmitted from Scrapie-infected sheep to cattle by feeding cattle with meat and bone meal prepared from sheep cadaver including the affected ones. Simultaneously, the sterilization temperature was reduced from 130 to 110°C and solvent extractions of fat were suppressed resulting finally in an insufficient inactivation of the agent and the possibility to cross the species barrier from sheep to cattle (for review see (Edenhofer *et al*., 1997)). After the UK-prohibition of feeding meat and bone-meal to ruminants in July 1988, the so-called ″feed-ban″, followed by the specified bovine offal ban (SBO) in November 1989, the number of BSE-affected cattles decreased dramatically yielding in about 50 confirmed newly infected BSE-cases per month in January 2002 in England (source: MAFF, UK). Some infected animals born after the feed ban might have been infected due to the vertical transmission of the infectious agent from the mother cow to the calf. Up to now, BSE-cases have been confirmed in 23 countries worldwide (Table I). Some of these cases have been imported from the UK as indicated in Table I.

Table I. Total numbers of confirmed BSE cases worldwide since 1987*

Country	Total number	Country	Total number
United Kingdom	181552	Japan	3
Ireland	877**	Czech. Republic	2
Portugal	597	Oman	2
France	529	Lichtenstein	2
Switzerland	406	Slovenia	2
Germany	151**	Austria	1
Spain	87	Finland	1
Belgium	69	Luxembourg	1
Italy	54**	Greece	1
Netherland	29	Falkland Islands	1
Denmark	8	Canada	1
Slovakia	5		

* http://ourworld-top.cs.com/j1braakman/BSE.htm (updated 26 january 2002)

** including imported cases from the UK

1.2. Sporadic, familial and transmitted (including iatrogenic) TSEs

Human prion diseases are fatal neurodegenerative disorders which have a sporadic, genetic or infectious origin. Genetic TSEs are autosomal dominant diseases caused by defined mutations within the prion protein gene (*Prn*-p). *Prn*-p represents a single-copy gene which is located on the short arm of chromosome 20 in humans (Sparkes *et al*., 1986). The 15 kb gene consists of two short, non-coding exons and a 2,4 kb exon 3 which contains the 759 bp coding region followed by a 1,64 kb non-translated 3′ region (Kretzschmar *et al*., 1986; Puckett *et al*., 1991). All mammalian genomes analysed so far contain a prion protein gene. In addition, the primary structure of PrP is highly conserved between different species (Schätzl *et al*., 1995).

Approximately 10-15% of all human prion diseases are familial. So far, three classical genetic human prion diseases have been described: Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS) (for review see (Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann and Aguzzi, 1997). Whereas 85% of all CJD cases occur sporadically in the absence of any mutations, FFI, GSS and 15% of CJD are dominantly inherited caused by several defined mutations within the *Prn*-p gene. Genetic

linkage has been identified for several amino acid substitutions in the core region of PrP as well as some insertions and one deletion within the octarepeat region of the molecule (Figure 1). Here, the spontaneous (*de novo*) conversion of PrP^{c} to a misfolded pathogenic isoform (PrP^{Sc}) in the absence of exogenous prions has been supposed (Young, 1999) but the exact mechanism by which these mutations provoke the conformational changes is still unknown. Destabilization of the PrP^{c} structure or effects on the thermodynamic stability of PrP caused by a few disease-specific amino acid replacements are suggested (Liemann and Glockshuber, 1999; Riek *et al*., 1998; Swietnicki *et al*., 1998; Zhang *et al*., 2000). For all known mutations within the *Prn*-p gene a genotyp-phenotyp association exists. In contrast to the classic CJD phenotype which features a rapidly progressing dementia, GSS is a more slowly progressing disease (incubation time for GSS: 3 to 10 years; for CJD: ~1 year) in which ataxia is the predominant clinical sign and dementia usually occurs at a later stage (Ghetti *et al*., 1995). Polymorphism at codon 129 of the prion protein gene is implicated both in susceptibility and phenotype of human prion diseases. In conjunction with the point mutation at residue 178, the valine/methionine polymorphism at position129 determines whether this mutation leads to the clinical phenotype of CJD or of FFI (Goldfarb *et al.*, 1992).

In infectious prion diseases such as Kuru as well as new-variant and iatrogenic CJD (transmission via treatment with contaminated human growth hormone or by surgery performed by physicians), exogenous PrP^{Sc} is thought to impress its conformation on endogenous host PrP^{c}, thereby generating more PrP^{Sc} in an auto-catalytic reaction (Prusiner *et al*., 1990). Sporadic CJD, which comprise most cases of the disease may result from rare, spontaneous conversion of wild-type PrP^{c} to PrP^{Sc} or from somatic mutations within the *Prn*-p gene (Cohen *et al*., 1994).

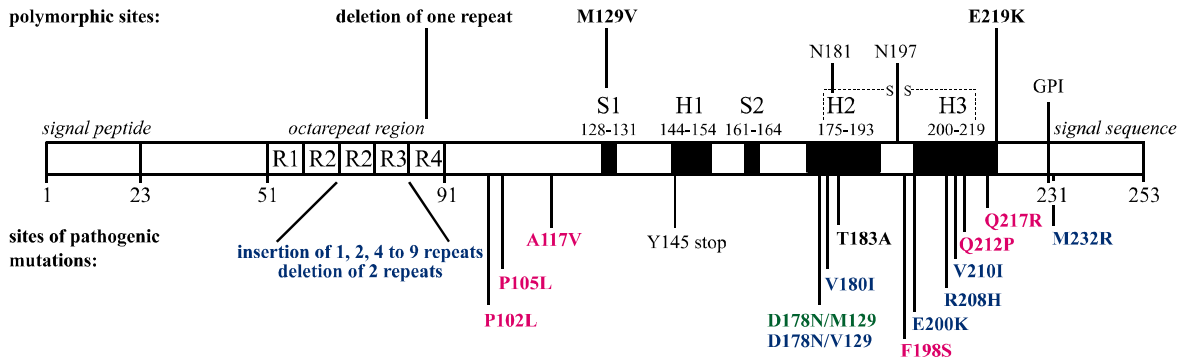


Fig. 1: PrP mutations, polymorphisms and structural elements of the human prion protein. Pathogenic mutations are shown below, polymorphisms and structural elements above the PrP scheme. H:-helix; S: -pleated sheet; N: asparagine-linked glycosylation site; GPI: glycosyl

phosphatidylinositol anchorage site; -S-S-: disulfide bridge between aa 179 and 214. Pathogenic mutations are marked coloured according to the different phenotypes occuring in genetic human prion diseases (GSS: pink; CJD: blue; FFI: green; dementia: bold black; PrP cerebral amyloid angiopathy (PrP-CAA) : black).

1.3. Transmission studies and occurence of new variant CJD

Despite extensive investigations of the bovine *Prn*-p gene in affected cattle, neither natural polymorphisms nor defined mutations within the PrP encoding gene have been associated with the susceptibility to BSE. Therefore, the most probable origin of BSE represents the transmission of the Scrapie agent to cattle via insufficiently treated Srapie-affected foodstuff feeded to cows, although sporadic BSE cases prior to the first reports of BSE cases cannot be excluded. A variety of transmission experiments have demonstrated that TSE agents are able to cross the species barrier dependent on the degree of homology in the *Prn*-p gene between infected donor and recipient. Several species such as cattle, goats, pigs, sheep, mice, mink, marmosets and macaques have been infected with the BSE agent by the parenteral route (Baker *et al*., 1993; Fraser *et al*., 1992; Lasmézas *et al*., 1996). The transmission by the oral route was only successful to cattle, goats, sheep, mice and mink. However, one of the most interesting questions concerns the transmission of this pathogenic agent to humans and the relationship between the BSE crises and the occurance of new variant CJD (vCJD) cases in March 1996 in Great Britain (Will *et al*., 1996). Until January 2002 113 definite and probable cases of vCJD have been reported in the UK (referred to the CJD surveillance unit in Edinburgh). Although BSE has mainly affected the UK, two definite cases and two probable cases of vCJD have been described in France even though these people have never been in the UK (Chazot *et al*., 1996; Deslys *et al*., 1997). All these patients show common features which differ from those noted for the classical form of CJD (sporadic and familial CJD). They were remarkable young with a mean age of 29 years in comparison to about 65 years observed for patients suffering from sporadic CJD. The duration of illness for vCJD (from the onset of the disease until death) was about 15 month, in contrast to the sporadic form of the disease with a duration period of 6 months. In addition, clinical aspects were different for both diseases. Sequencing of the open reading frames of the *Prn*-p genes of vCJD patients revealed no mutations and all patients were homozygous for methionine at codon 129 (Zeidler *et al*., 1997). However, it cannot be concluded that heterozygocity at this codon protects against infection and leads to vCJD immunity. It seems that people who are homozygous for methionine at this position are more prone to develop the disease than people who are heterozygous. In the caucasian population 40% are homozygous for methionine, 50%

heterozygous for methionine/valine and 10% homozygous for valine (Collinge, 1999). Very recently, an altered frequency of the human leukocyte antigen (HLA) class-II type DQ7 was observed in patients suffering from vCJD compared to control persons (sporadic or familial CJD-patients or non-infected people) revealing that the HLA-DQ7 antigen is clearly underrepresented in patients with vCJD but not in those with the classical form of disease and therefore seems to protect against vCJD (Jackson *et al*., 2001a). This apparently protective genetic factor should aid differential diagnosis and may have important implications for the understanding of host susceptibility towards infections with BSE prions.

One of the most amazing features represents the neuropathology of vCJD patients. The brains exhibit amyloid plaques surrounded with vacuoles, so-called florid plaques, which are similar to those described for some Kuru patients (in addition to kuru-type plaques found in the brains of those affected persons) but which have never been observed in the brains of classical CJD patients. The location and distribution of florid plaques is similar for all vCJD patients examined so far. The most affected locations in the brain are cerebrum and cerebellum. Spongiosis, astrocytosis, gliosis and neuronal loss are detected mainly in basal ganglia and thalamus. It is noteworthy that PrP depositions consist of proteinase K resistant material and are observed in the absence of confluent spongiform changes in the surrounding neurophils (Chazot *et al*., 1996; Will *et al*., 1996). It has been supposed that the origin of vCJD happened during the late eighties and the beginning nineties where presumably high amounts of BSE-contaminated material has entered the food chain. This might explain why most of the affected people originate from the UK, the country in which the BSE epidemic appeared. In addition, experminantal data i.e. transmission studies with transgenic animals carrying the human *Prn*-p gene (Hill *et al*., 1997; Scott *et al*., 1999), molecular analysis of prion strain variation (Collinge *et al*., 1996) and inoculation experiments with mice infected with BSE- and vCJD-prions showing the same lesion profiles and incubation times (Bruce *et al*., 1997; Lasmezas *et al*., 2001) demonstated that the same agent causes both BSE and vCJD.

2. Prion, the causative agent of TSEs

Prions represent a new class of pathogenic agents in which a protein denoted PrP^{Sc} appears to be the sole component ot the infectious particle causing transmissible spongiform encephalopathies in humans and animals. The mechanism of disease propagation is thought to involve the interaction of PrP^{Sc} with its normal cellular isoform PrP^{c}. Thereby, PrP^{c} undergoes a posttranslational structural conversion resulting finally in the abnormally folded

PrP^{Sc}. Prion diseases have been associated with the accumulation of this misfolded protein and its neurotoxic effects, however, it remains unclear whether the PrP^{c} "loss of function" represents a crucial factor.

2.1. The physiological role of PrP^{c}

The physiological role of the cellular prion protein is poorly understood. In order to characterize the function of PrP^{c}, a series of PrP-deficient ($PrP^{0/0}$) mice have been generated by several groups differing in their strategy to knock-out the PrP encoding gene. Surprisingly, some of these mouse lines developed completely normal without any visible phenotype (Bueler *et al*., 1992; Lledo *et al*., 1996; Manson *et al*., 1994). These results were amazing since PrP is expressed predominantly in the brain and since the *Prn*-p gene is highly conserved among species (85-97% among mammals (Gabriel *et al*., 1992)). Therefore researchers expected dramatic physiological deficiencies in those animals. In contrast, two further independently generated mouse lines in which the PrP gene has been ablated by other strategies revealed late-onset ataxia associated with a loss of cerebellar Purkinje cells (Moore *et al*., 1999; Sakaguchi *et al*., 1996). These observations have been interpreted as evidence that PrP might play a role in the long-term survival of Purkinje cells. However, the discovery of a PrP-like gene, *Prn*-d, which is located 16 kb downstream of the *Prn*-p gene and encodes for doppel (Dpl) revealed that the loss in Purkinje neurons and ataxia in these mice were caused by Dpl overexpression, an unexpected consequence of the target inactivation of the *Prn*-p locus (Moore *et al*., 1999). Other reports using $PrP^{0/0}$ mice described a role of PrP^{c} in synaptic processes (Collinge *et al*., 1994) or described an alteration in circadian activity rythms and sleep (Tobler *et al*., 1996).

In addition, an involvement of PrP in the metabolism of copper has been reported (Hornshaw *et al*., 1995). Several studies indicated that PrP^{c} can bind copper (cooperative binding of four Cu^{2+} per PrP^{c} molecule) via the histidine-containing octapeptide repeats in the amino-terminal domain of the prion protein (Brown *et al*., 1997; Viles *et al*., 1999). However, the exact functional relationship between copper and PrP^{c} is still unclear. Recently, a Cu/Zn superoxide dismutase (SOD) activity has been described for recombinant PrP and for PrP immunoprecipitated from mouse brain tissues (Brown *et al*., 1999). These results suggest that PrP^{c} harbors an enzymatic activity that depends on the incorporation of copper as an essential cofactor and finally might contribute to cellular resistance against oxidative stress.

In contrast, very recent data have shown that PrP^c might play a role in signal transduction and triggers cell signaling increasing the phosphorylation levels of the tyrosine kinase Fyn (Mouillet-Richard, 2000). Caveolin-1 and Clathrin were described as possible intermediate factors which might connect PrP^c on the outer membrane to the intracellular kinase Fyn. However, the ligand i.e. a transmembrane receptor which might be responsible for the generation of the signal and which might mediate the PrP^c dependent activation of Fyn has not been identified by this group (Mouillet-Richard, 2000). Two other PrP interacting partners were found which are implicated in neuronal signaling processes, the neuronal phosphoprotein synapsin Ib and the adaptor protein Grb2 (Spielhaupter and Schätzl, 2001). These findings further strengthen the putative role of the prion protein in signal transduction.

Despite of numerous studies suggesting various possibilities for the physiological role of PrP^c, its exact function as a cellular membrane-localized protein is still unclear. The only proven phenotype of PrP^c remains its absolute necessity for an organism towards a PrP^{Sc} infection since PrP-ablated mice were totally resistant towards an infection with mouse-adapted Scrapie strains (Bueler *et al*., 1993).

2.2. Biochemical and structural features of PrP^c

Mammalian PrP genes encode proteins which contain a N-terminal signal peptide, a series of five proline- and glycine-rich octapeptide repeats, a central highly conserved hydrophobic segment, and a C-terminal hydrophobic region that represents a signal for the addition of a glycosyl phosphatidylinositol (GPI) anchor (Figure 1). PrP^c is synthesized in the rough endoplasmic reticulum (ER) and transits through the Golgi on its way to the cell surface. During biosynthesis the PrP precursor becomes processed as follows: (i) the N-terminal signal peptide is removed, (ii) two N-linked oligosaccharide chains are added, (iii) a single disulfide bond is formed and (iv) a GPI anchor becomes attached (Endo *et al*., 1989; Stahl *et al*., 1987; Turk *et al*., 1988). The N-linked oligosaccharide chains, of the high mannose-type, are initially added within the ER and are subsequently modified in the Golgi resulting in an endoglycosidase H resistance. The GPI anchor is added in the ER after cleavage of the hydrophobic C-terminal signal sequence. After its passage through the secretory pathway PrP^c remains on the cell surface where it is attached to the plasma membrane via the GPI-moiety.

The conversion of PrP^c to its pathogenic, infectious isoform, PrP^{Sc}, is accompanied by conformational changes. Spectroscopic studies revealed that PrP^c has a high α-helical content

whereas PrP^{Sc} is rich in β-sheets (Table II). These two isoforms also differ biochemically with PrP^{Sc} displaying reduced solubility in non-denaturing detergents and partial resistance towards proteinase K digestion (Meyer *et al*., 1986; Oesch *et al*., 1985). Whereas PrP^{c} is completely sensitive to proteinase K digestion, PrP^{Sc} is degraded to a truncated highly infectious form termed PrP27-30 which lacks about 60 amino acid residues at the N-terminus compared to PrP^{Sc} (Table II). Which form of PrP is the dominant one in an affected individual is thought to depend on the presence of cellular proteinases in the individual tissue or organ.

Table II. Characteristics of PrP^{c}, PrP^{Sc} and PrP27-30

Characteristics	PrP^{c}	PrP^{Sc}	PrP27-30
Secondary structure			
- α-Helix	42%	30%	21%
- β-Sheet	3%	43%	54%
Proteinase K status	sensitive	partially resistant	resistant
Infectivity status	non infectious	infectious	infectious

Recently the three-dimensional structure of soluble, recombinant PrP^{c} from mouse (Riek *et al*., 1996; Riek *et al*., 1997), Syrian hamster (Donne *et al*., 1997), human (Zahn *et al*., 2000; Zhang *et al*., 2000) and cattle (Lopez Garcia *et al*., 2000) (Figure 2) have been determined by nuclear magnetic resonance (NMR) spectroscopy, revealing that the core region of the protein forms a globular domain with three α-helices and a short two-stranded antiparallel β-sheet, while the N-terminal segment remains flexibly disordered in solution. The three-dimensional structures of prion proteins of different species are quite similar. However, the highest degree in structural identity has been observed between human and bovine PrP (Lopez Garcia *et al*., 2000). The role of the flexible N-terminal segment is not yet fully understood: a role in binding/uptake of copper ions which might stimulate the endocytosis of PrP (Pauly and Harris, 1998) or the interaction with a ligand required for signal transduction (Shmerling *et al*., 1998) have been suggested.

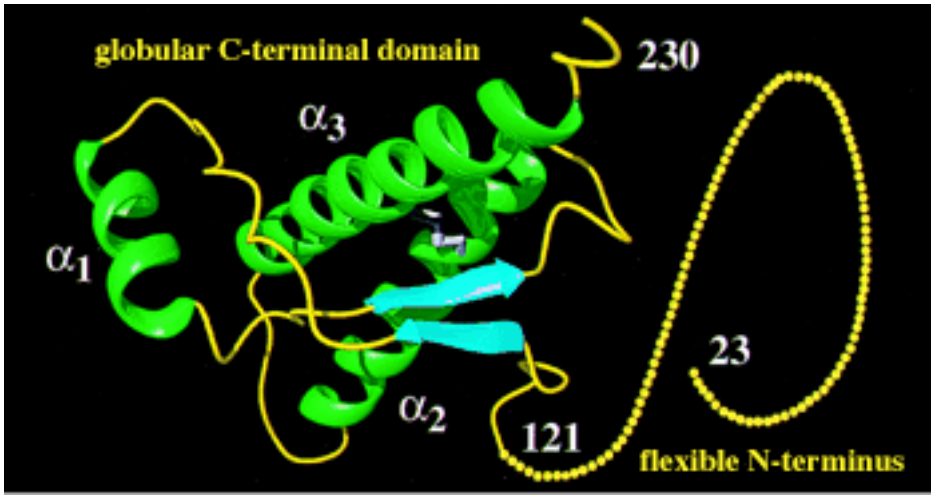


Fig. 2: NMR structure of recombinant, soluble bovine PrP from residue 23 to 230 modified from (Lopez Garcia *et al*., 2000). The globular domain extending from aa 125-227 contains three α-helices (green) and a short antiparallel β-strand (cyan). Helix 2 and helix 3 are linked by one disulfide bond. Segments with non-regular secondary structures within the core region are in yellow, and the flexible disordered "tail" of residues 23-121 is represented by 108 yellow dots for each amino acid residue. (The native mature boPrP stretches from aa 25 to 242. Lopez-Garcia published a structure of boPrP from aa 23 to 230).

Very recently, the crystal structure of the human prion protein reveals dimer formation resulting from the three-dimensional swapping of the C-terminal helix 3 and the rearrangement of the disulfide bond (Knaus *et al*., 2001). An interchain two-stranded antiparallel β-sheet is generated at the interface of the dimer by residues located in helix 2 in the monomeric NMR structure (Knaus *et al*., 2001). As suggested in the nucleation dependent polymerization model of prion propagation, the occurence of such PrP-dimers could accelerate the generation of a nucleus that might act as a seed for the formation of highly ordered PrP^{Sc} aggregates.

2.3. Replication mechanisms of prions ("protein-only" and "nucleation dependent polymerization" hypothesis)

In the mid-sixties Alper and Griffith suspected that the scrapie agent could replicate in the absence of any specific nucleic acid (Alper *et al*., 1967; Alper *et al*., 1966; Griffith, 1967). 15 years later Stanley Prusiner stamped the term "prion" (proteinaceous infectious particle) and mentioned in his "protein-only" hypothesis that the prion is an infectious protein that causes the disease (Prusiner, 1982). In contradiction to classical agents of infectious diseases such as

viruses, bacteria and fungi, prions are stated to replicate in the absence of any nucleic acid. The replication scheme is quite simple: an infectious PrP^{Sc} molecule binds to the endogeneous PrP^{c} most likely in the endocytotic pathway of the Scrapie-infected cell forming a heterodimer. This event created the term "heterodimer" model. Due to the direct interaction of both molecules, PrP^{c} is forced to undergo structural changes yielding in an increase in its β-sheet content and thereby is converted into PrP^{Sc}. In a chain reaction further PrP^{c} molecules are converted into the pathogenic, but thermodynamically more stable isoform resulting in prion propagation and finally astrocytosis, gliosis, vacuolization and apoptosis. For this striking and completely new replication mechanism, S.B. Prusiner received the Nobel Prize for Medicine in 1997. Another favored model, the nucleation dependent polymerization or crystal seed model claims the existence of a nucleus consisting of a few PrP^{Sc} molecules (Lansbury and Caughey, 1995). Different crystals originate from different species or strains. Here, the most important factor for prion propagation represents the similarities between PrP^{c} and one PrP^{Sc} monomer in the seed. The higher the similarities between both molecules the better the polymerization proceeds. This model offers an explanation for the existance of different scrapie strains within the same species and the existance of species barriers. Although the "protein-only" hypotheses (encompassing the heterodimerization and the nucleation-dependent polymerization model) are favoured by the majority of scientists in the field, there are further hypotheses such as the virino and the viral ones that assume the existance of a so far undiscovered nucleic acid or virus specific for the Scrapie agent (Dickinson and Outram, 1988; Dickinson *et al*., 1989; Manuelidis *et al*., 1988; Özel *et al*., 1994).

3. Trafficking of PrP and the role of a cell surface receptor

PrP^{c} is a cell-surface glycoprotein which is expressed in the rough endoplasmic reticulum (ER) of neuronal (or other scrapie-infectable) cells. Recently, Prusiner and Lingappa localized three forms of PrP at this compartment which differ in their topology: the carboxy terminal transmembrane protein ^{Ctm}PrP, the amino terminal transmembrane protein ^{Ntm}PrP and the secretory form of the prion protein ^{Sec}PrP (Hegde *et al*., 1998). In contrast to ^{Sec}PrP, ^{Ctm}PrP and ^{Ntm}PrP span the membrane once via a conserved, hydrophobic domain, ^{Ctm}PrP with the C-terminus and ^{Ntm}PrP in the opposite orientation with the N-terminus directed towards the lumen of the ER (Hegde *et al*., 1998). It has been proposed that both membrane-spanning

forms occur as key intermediates in infectious and inherited prion diseases but the exact mechanism by which these forms are produced is still unclear. The classical isoform, ^{Sec}PrP, traffics through the secretory pathway via the golgi apparatus and secretory granules to the cell surface where it is GPI-anchored to the plasma membrane (Figure 3). The subcellular trafficking of PrP^{c} has been widely studied employing transfected cell lines that express the protein. The results demonstrate that PrP^{c} does not remain on the cell surface, but rather constitutively cycles between the plasma menbrane and an endocytic compartment (Shyng *et al*., 1993). This endocytic pathway is of great interest because it is thought that the conversion of PrP^{c} to PrP^{Sc} takes place in certain endocytic compartments. Clathrin-coated pits and vesicles are suggested to be the morphological structures responsible for the endocytic uptake of PrP^{c} (Shyng *et al*., 1994). This observation is surprising for a GPI-anchored protein like PrP^{c} that lacks a cytoplasmic domain which is thought to interact directly with adapter proteins and clathrin. Therefore, Harris postulated the existence of a PrP^{c} receptor, a transmembrane protein with a coated-pit localization signal in its cytoplasmic domain and a PrP-binding site in its extracellular domain, which mediates the endocytosis of PrP and direct the complex into clathrin-coated pits (Harris *et al*., 1996). In contrast, it has been speculated that the internalization is mediated receptor-independent by caveolae-like membranous domains (Anderson, 1993; Vey *et al*., 1996). Other reports proposed that PrP is associated to cholesterol and sphingolipid enriched domains (Gorodinsky *et al*., 1995; Taraboulos *et al*., 1995) and that different kind of such specialized microdomains ("rafts") exist on the surface of neuronal cells in which PrP^{c} might be enriched (Madore *et al*., 1999).

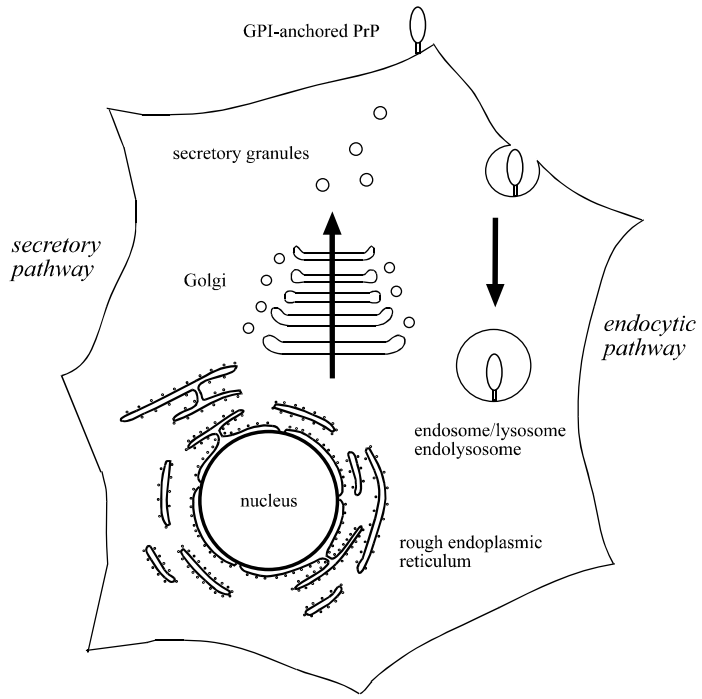


Fig. 3: Trafficking of cellular PrP. PrP^{c} is synthesized in the rough endoplasmic reticulum and after the passage through the golgi apparatus and secretory granules, it reaches the cell surface where it is anchored to the plasma membrane via its GPI-moiety. PrP^{c} becomes internalized via clathrin-coated pits or caveolae-like membranous domains and enters the endocytic pathway encompassing endosomes, lysosomes and endolysosomes, compartments in which the $PrP^{c} \rightarrow PrP^{Sc}$ conversion process might occur.

The presence of a specific so far unidentified cell-surface receptor for PrP with a molecular weight of 66 kDa has been deduced by means of complementary hydropathy (Martins *et al*., 1997). Simultaneously, the human 37-kDa laminin receptor precursor (LRP), the precursor of the 67 kDa high-affinity laminin receptor (LR), was identified as an interaction partner of PrP^{c} in a yeast two-hybrid screen (Rieger *et al*., 1997). Several reasons exist which favour LRP/LR as a possible cellular prion protein receptor (for review see (Gauczynski *et al*., 2001a; Rieger *et al*., 1999), Chapter II, respectively). (i) Both molecules are well-represented on the surface of neuronal cells (Rieger *et al*., 1997). (ii) LRP is clearly increased in several organs and tissues of Scrapie-infected mice and hamsters such as brain, spleen and pancreas compared to uninfected control animals revealing a correlation between the LRP level and the occurance of infectivity and PrP^{Sc} accumulation in those organs (Rieger *et al*., 1997). (iii) Mapping analyses of the LRP/PrP interaction site performed in the yeast two-hybrid system

demonstrated that the laminin-binding site encompassing residues 161 to 180 (Castronovo *et al*., 1991b) can also function as a binding domain for PrP^{c} (Hundt *et al*., 2001; Rieger *et al*., 1997) (Chapter IV). The 37-kDa LRP is thought to be the precursor of the 67-kDa high-affinity LR which was first isolated from solid tumors and exhibit a high binding capacity to laminin, a glycoprotein of the extracellular matrix mediating cell attachment, movement, differentiation and growth (Beck *et al*., 1990; Rao *et al*., 1989; Rao *et al*., 1983). The mechanism how the high-molecular LR is formed by the 37-kDa precursor is still unclear. Heterodimerization caused by the interaction of LRP with an additional so far unknown protein or acylation of the precursor has been suggested (Buto *et al*., 1998; Castronovo *et al*., 1991a). In addition, yeast two-hybrid analyses revealed that LRP does not interact with itself disproving the theorie that the high-molecular form of the receptor represents a homodimer of the 37-kDa form (Hundt *et al*., 2001) (see Chapter IV). Like PrP^{c}, LRP/LR is well conserved throughout evolution showing a high degree of homology among mammalian species (Rao *et al*., 1989). Within the life cycle of prions, we suggest that this surface-located transmembrane protein may be involved in the receptor-mediated endocytosis of the GPI-anchored PrP^{c} and potentially of exogenous PrP^{Sc}. In order to further characterize the role of LRP/LR as a binding partner for the prion protein, cell-binding/internalization assays for PrP were performed with various cultured cell lines revealing the LRP/LR-dependent internalization of recombinant PrP by neuronal and non-neuronal cells (Gauczynski *et al*., 2001b) (see Chapter III). Two binding sites were identified: a direct binding domain identified by yeast two-hybrid mapping studies and a heparansulfate proteoglycan (HSPG)-dependent interaction site identified by cell-binding assays using a series of recombinant PrP peptides as well as various mutated LRP molecules (Hundt *et al*., 2001) (see Chapter IV). Furthermore, we found that PrP co-localizes with LRP/LR at the surface of neuroblastoma cells and with LRP on transfected BHK cells co-expressing both proteins. On the basis of all these data, we conclude that LRP/LR acts as the cellular prion protein receptor playing an essential role in the normal cell cycle of PrP by mediating its endocytosis. It might be conceivable that the internalization process of PrP^{Sc} proceeds also receptor-dependent. After the entry into the nerve cell, either directly or receptor-dependent, the pathogenic PrP isoform comes in contact with PrP^{c} probably during the endocytotic pathway involving endosomes, lysosomes and endolysosomes, compartments in which finally the conversion of PrP^{c} and the aggregation of PrP^{Sc} is thought to take place. A first contact between PrP^{c} and PrP^{Sc} on the cell surface is also conceivable. Here a PrP^{c} and LRP/LR mediated internalization of PrP^{Sc} is imaginable

(as shown in the diploma thesis from Susanne El-Gogo, February 2002, "Bindungs- und Internalisierungsstudien des murinen Scrapie prion proteins PrP27-30 an Säugerzellen mit Hilfe des Semliki-Forest-Virus Systems" performed in the Weiss laboratory, Gene Center, Munich). The involvement of an additional, so far unknown protein termed protein X (Telling *et al*., 1995) which might represent a molecular chaperone such as Hsp60 (Edenhofer *et al*., 1996) in the conversion process cannot be excluded.

Due to the capability of LR to bind laminin and elastin, two proteins of the extracellular matrix, the interaction with PrP may induce a signal essential in cell survival. Recently, it has been shown that PrP^{c} might play a role in signal transduction by activating the intracellular tyrosine kinase Fyn and might trigger cell signaling (Mouillet-Richard, 2000). However, the ligand i.e. a transmembrane receptor which might be responsible for the generation of the signal and which might mediate the PrP^{c} dependent activation of Fyn has not been identified so far (Mouillet-Richard, 2000). The plasma membrane-spanning LRP/LR might represent such a protein which connect, either directly or indirectly via HSPGs, the GPI-anchored PrP with the intracellular plasma-membrane-associated Fyn kinase (Hundt *et al*., 2001).

The identification of the cellular prion receptor might not only allow a better understanding of the cellular processes in normal and in Scrapie infected cells, but might also represent a point of attack for anti-prion drugs interfering with the receptor mediated binding and endocytosis of PrP^{c} and/or PrP^{Sc}. A possible strategy would be the blockage of the PrP^{c} /PrP^{Sc} entry by using LRP-specific antibodies which are raised against the PrP binding domains (Figure 4).

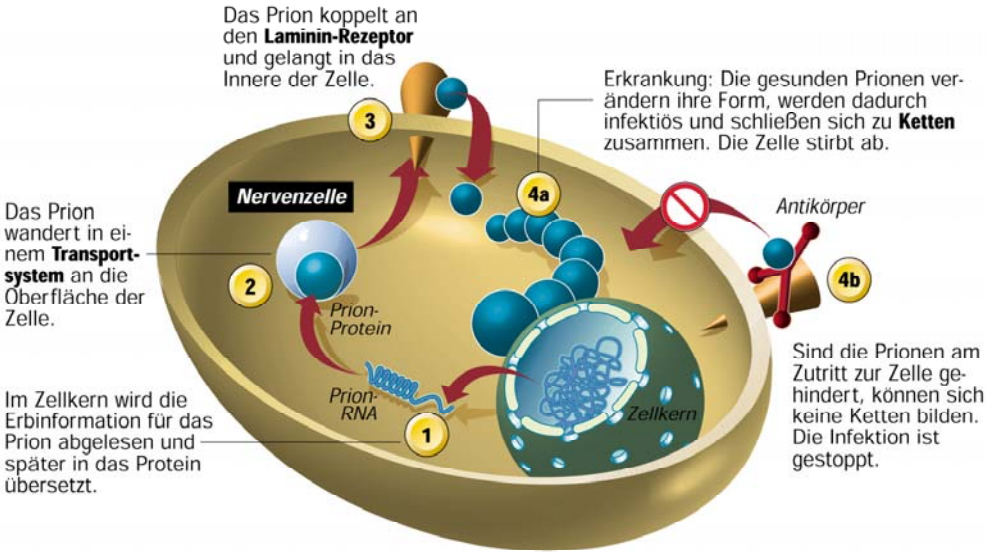


Fig. 4: Life cycle of the prion protein (1-3) and the anti-prion effect of antibodies raised against the cellular prion receptor (4). PrP^{c} is synthesized within the neuronal cell and transported to the plasma membrane via the secretory pathway. After its exposure to the cell surface, PrP^{c} binds to its receptor and becomes internalized. The conversion of the normal prion protein to its infectious form might occur spontaneously or PrP^{Sc} mediated within compartments of the endocytic pathway of the cell leading to PrP^{Sc} aggregation and finally to cell death. Antibodies which specifically bind to this transmembrane receptor protein prevent the entry of the cellular and presumably the pathogenic prion protein and therefore protect the cell against the infection. (The graphic is adapted from FOCUS 46/2001, p.176 and therefore labeled in German. We thank the FOCUS Magazine for providing us with this image).

4. Therapeutics for prion diseases

Until now, no effective therapy is available that cure a patient or an animal suffering from a manifested TSE. A variety of classical drugs used in human therapy have been tested but among them, only a few delay the appearance of the disease in animals experimentally infected with PrP^{Sc}. The research in this field is mainly hampered by the uncertainties remaining about the replication mechanisms of the TSE agent and furthermore the exact mode of action of life-prolonging therapeutic drugs is widely unknown.

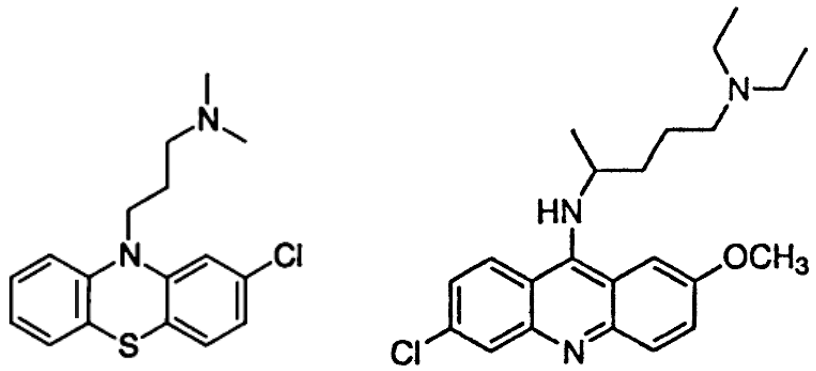
Polyanions such as heteropolyanion 23 (HPA-23), Dextran Sulfate 500 (DS-500), heparin and pentosan polysulfate (SP54) have been shown to be efficient in experimental Scrapie. Although HPA-23 and DS-500 probably act on the early replication phase of the agent in the lymphoreticular system (LRS), their application is limited due to a toxicity at therapeutic doses. In addition, they are only effective when administered at the time of infection (Ehlers and Diringer, 1984; Kimberlin and Walker, 1983; Kimberlin and Walker, 1986) (in detail see

Chapter II). To date, the polyanion pentosan polysulfate (SP54) represents one of the most promising candidates in the field of anti-scrapie drugs; first, SP54 was effective at extreme low doses and secondly, it was clearly less toxic. SP54 is thought to be effective during the very early events of pathogenesis by interfering with the uptake of PrP^{Sc} by nerve endings (Farquhar *et al*., 1999; Ladogana *et al*., 1992) (see Chapter II). Preliminary experiments suggest, that SP54 might interfere with the PrP^{Sc}/LRP-LR interaction process on the cell surface resulting in the blockage of binding and presumably internalization of the Scrapie prion protein (as shown in the diploma thesis from Susanne El-Gogo, February 2002, "Bindungs- und Internalisierungsstudien des murinen Scrapie prion proteins PrP27-30 an Säugerzellen mit Hilfe des Semliki-Forest-Virus Systems" performed in the Weiss laboratory, Gene Center, Munich).

Congo Red, normally used as a diagnostic dye for amyloids, inhibits PrP^{Sc} accumulation and prion replication in chronically, scrapie-infected mouse neuroblastoma (ScN2a) cells (Caughey *et al*., 1993a) and in hamsters inoculated with the pathogenic agent (Ingrosso *et al*., 1995) (see Chapter II). Congo Red may also act during the early phases of infection; its ability to insert into amyloid proteins suggests that it binds directly to PrP^{Sc} and therefore, interfers with PrP^{Sc} formation and replication (Caspi *et al*., 1998).

Amphotericin B (AmB), widely used for the treatment of fungal infections, and its derivative MS-8209 belongs to the group of polyene macrolide antibiotics which increase the incubation period of Scrapie infected rodents probably by the direct prevention of PrP conversion or interference with the uptake of PrP^{Sc} (Adjou *et al*., 1995; Pocchiari *et al*., 1989) (see Chapter II). Whereas the application of AmB is hardly restricted due to its acute nephrotoxicity, the derivative MS-8209 shows at least a five times lower toxicity and a higher solubility without a loss of efficiency in delaying the disease and PrP^{Sc} accumulation (Adjou *et al*., 1999) .

Very recently, potential anti-prion effects of quinacrine and chlorpromazine, tricyclic derivatives of acridine and phenothiazine, were observed in ScN2a cells (Korth *et al*., 2001). They represent a new class of pharmacotherapeutics for prion diseases consisting of a tricyclic ring structure and an aliphatic side chain at the middle ring moiety (Figure 5). The mechanism by which these reagents inhibit PrP^{Sc} formation is unknown, but both compounds require only a few days of treatment before PrP^{Sc} disappear in chronically infected N2a cells. Since quinacrine and chlorpromazine have already been used in humans as anti-malarial and anti-psychotic drugs and are known to penetrate the blood-brain barrier, they are auspicious candidates for the treatment of CJD and other prion diseases.



chlorpromazine **quinacrine**

Fig. 5: Structure of the anti-prion compounds chlorpromazine and quinacrine. They consist of a tricyclic scaffold with an aliphatic side chain extending from the middle ring moiety.

Recent data showed that exposure of Scrapie infected N2a cells to anti-PrP antibodies or to the enzyme phosphatidylinositol-specific phospholipase C (PIPLC) which cleaves PrP^{c} off the cell surface causes rapid loss of PrP^{Sc} and even cures the chronically Scrapie-infected cultures (Enari *et al*., 2001; Peretz *et al*., 2001). Therefore, the level of PrP^{Sc} is determined by the rates of its formation from PrP^{c} and its degradation. Expression of anti-prion protein antibodies in transgenic mice resulted in the prevention of pathogenesis of Scrapie prions introduced by intraperitoneal injection in the spleen and brain (Heppner *et al*., 2001). These observations encourage the use of antibodies in the prevention and treatment of prion diseases.

A better understanding of the cellular processes regarding the uptake of the Scrapie agent into the cell and the exact conversion mechanisms within the cell might be necessary for the efficient application of therapeutic drugs to prevent the PrP^{Sc} replication, PrP^{Sc} accumulation and cell death. Therefore, the identification of a cellular receptor that mediates the binding and internalization of PrP^{c}/PrP^{Sc} is important to develop compounds such as specific antibodies which block the early step of infection i.e. the entry of PrP^{c}/PrP^{Sc} into the cell. In addition, a precise transport of such therapeutics to the affected tissues might be essential. PEGylated polycyanoacrylate nanoparticles have been suggested as a vector for an efficient drug delivery in prion diseases leading to a higher uptake by the spleen and the brain which both represent target tissues of PrP^{Sc} accumulation in Scrapie-infected animals (Calvo

et al., 2001). Those particles are very useful for anti-prion drugs which do not pass the blood-brain barrier.

5. Generation of recombinant proteins by the Semliki Forest virus (SFV) system in cultured cells

Cell culture models are useful tools to study the biology of PrP^{c}, to get a better understanding of the nature of the pathogenic isoform PrP^{Sc} including its propagation and to screen for potential therapeutic drugs and diagnostic tools.

In this work the Semliki Forest virus (SFV) expression system was utilized to (i) generate recombinant soluble PrP (Chapter V), (ii) synthesize human PrP mutants which are associated with familial prion diseases (Chapter V) and (iii) perform binding and internalization assays for the identification of the cellular prion protein receptor (Chapter III and IV). While prokaryotic and yeast expression systems are highly efficient and easy to use, expression systems for higher eukaryotic cells are still hampered by difficulties such as insufficient levels of protein production, restriction in host range and/or complexity of the system. Although the baculovirus system is one of the most efficient expression systems regarding protein production, its application, however, is strictly restricted to insect cells which are known to glycosylate proteins different from animal cells. Therefore, in order to generate correctly processed and glycosylated proteins such as prion proteins, the SFV system offers a variety of advantages which overcome those obstacles. The SFV system supplies a multitude of advantages for the expression of recombinant proteins in mammalian cells: (i) a large-scale production for up to 72 hours post transfection/infection, (ii) a broad host range, (iii) modifications such as glycosylations and (iv) the easy and fast transfection procedure with *in vitro*-transcribed RNA (for review see (Tubulekas *et al*., 1997)). An efficient production of a series of cytoplasmic, membrane-associated and secreted proteins e.g. the mouse dihydrofolate reductase (a cytoplasmic protein), the human transferrin receptor (a membrane protein) and the chicken lysozyme (a secreted protein) has been demonstrated (Liljestrom and Garoff, 1991).

The Semliki Forest virus is an enveloped RNA virus that belongs to the alphavirus group of the familiy Togaviridae and infects a variety of mammalian and insect cells. SFV expression vectors are based on a cDNA copy of the viral genome which consists of capped and polyadenylated single-stranded RNA of positive polarity and encodes its own RNA polymerase. Here, viral structural genes are deleted and replaced by the gene of interest. The

recombinant plasmid serves as a template for *in vitro* synthesis of recombinant RNA which is then transfected with an efficiency of up to 100 % into the cell. Due to the viral replicase which leads to an efficient production of recombinant RNA within the cell, a high-level synthesis of the foreign protein proceeds (for review see (Tubulekas *et al*., 1997)). Another possibility is the infection of cell lines with recombinant high-titer virus particles. Different cell lines and primary cell cultures were infected with such alphaviral particles resulting in the high-level production of a variety of CNS-related recombinant proteins such as Presenilin, APP, COX-2, Neurokinin, Dopamine, Serotonin etc. (for review see (Lundstrom, 1999)). The ability to infect primary neurons has made SFV an useful tool in neurobiology (Cook *et al*., 1996; Olkkonen *et al*., 1993; Simons *et al*., 1995; Ulmanen *et al*., 1997).

The SFV cell culture system is suitable to express high amounts of glycosylated wild-type and mutant disease-associated prion proteins in cultured mammalian cells on the one hand and allows localization and functional studies of recombinant proteins and their relationship to other cellular components on the other hand. Furthermore, the possibility to infect or transfect cell cultures simultaneously with several recombinant virus stocks or RNAs offers the investigation of protein-protein interaction. (Co-)localization studies and cell-binding assays with transfected or co-transfected cells overexpressing different proteins, separately or simultaneously, were performed to analyse the correlation between PrP and its cellular receptor LRP/LR to get a better understanding in the biology of prion proteins and the cellular processes in which they are involved (Gauczynski *et al*., 2001b; Hundt *et al*., 2001). In addition, recombinant SFV particles expressing various *Prn*-p genes were used to inoculate PrP-deficient $PrP^{0/0}$ mice for the generation of a series of monoclonal antibodies raised against human prion proteins (Krasemann *et al*., 1996a; Krasemann *et al*., 1996b; Krasemann *et al*., 1999).

CHAPTER II

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

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D.Therapeutics

E. Nucleic Acids

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

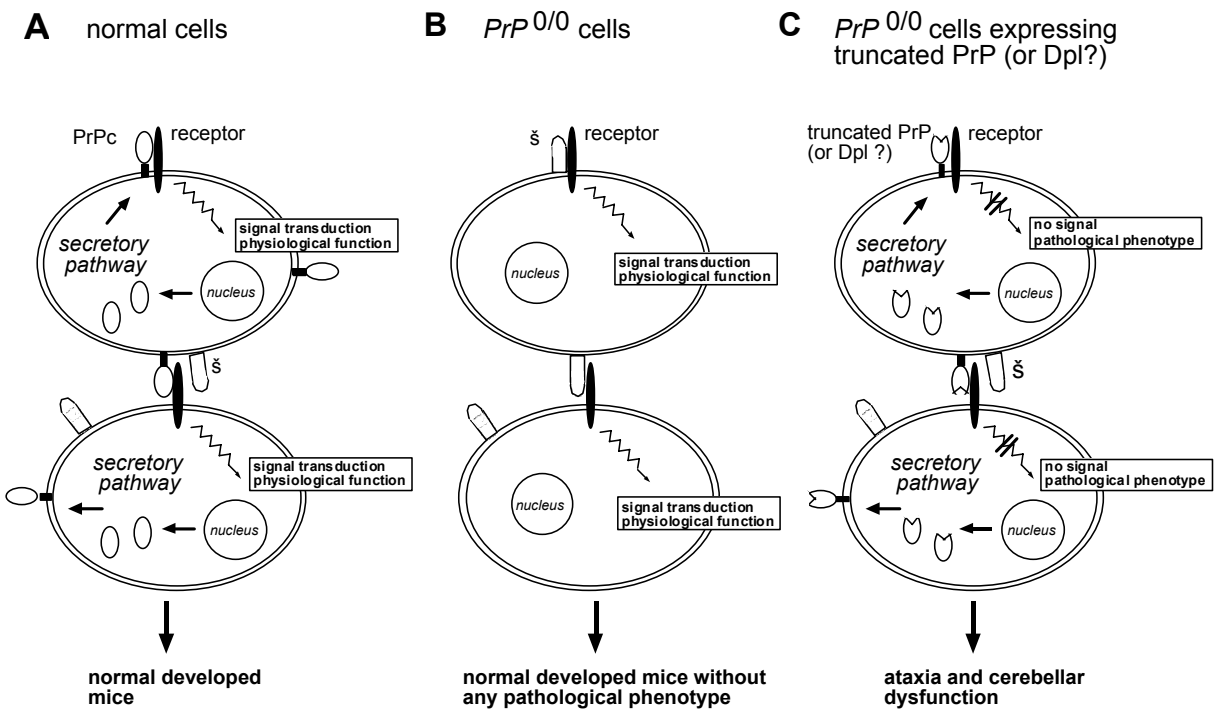

Fig. 1: Model of PrP^c- and receptor-mediated signal transduction. In the normal cell, PrP^c and receptor molecules from the same cell or from different cells can interact and promote signal transduction (A). The same signal might be elicited by the binding of a conjectural protein designated π, which 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).

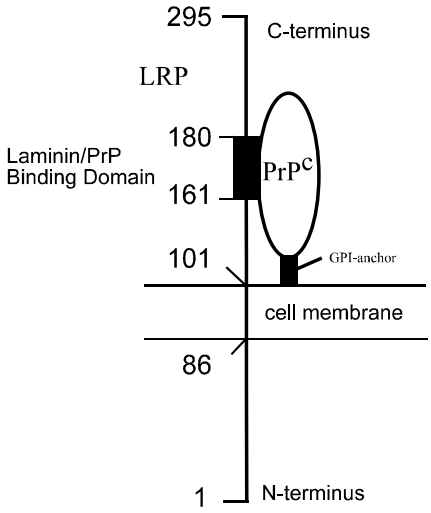

Fig. 2: Schematic view of the prion protein (PrP) and the 37 kDa laminin receptor precursor (LRP) on the surface of a scrapie-infectable cell. PrP is anchored by GPI (Blochberger *et al*., 1997) and is thought to colocalize with LRP. The putative transmembrane region of LRP stretches from aa 86 to aa101 (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.

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)

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.

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.

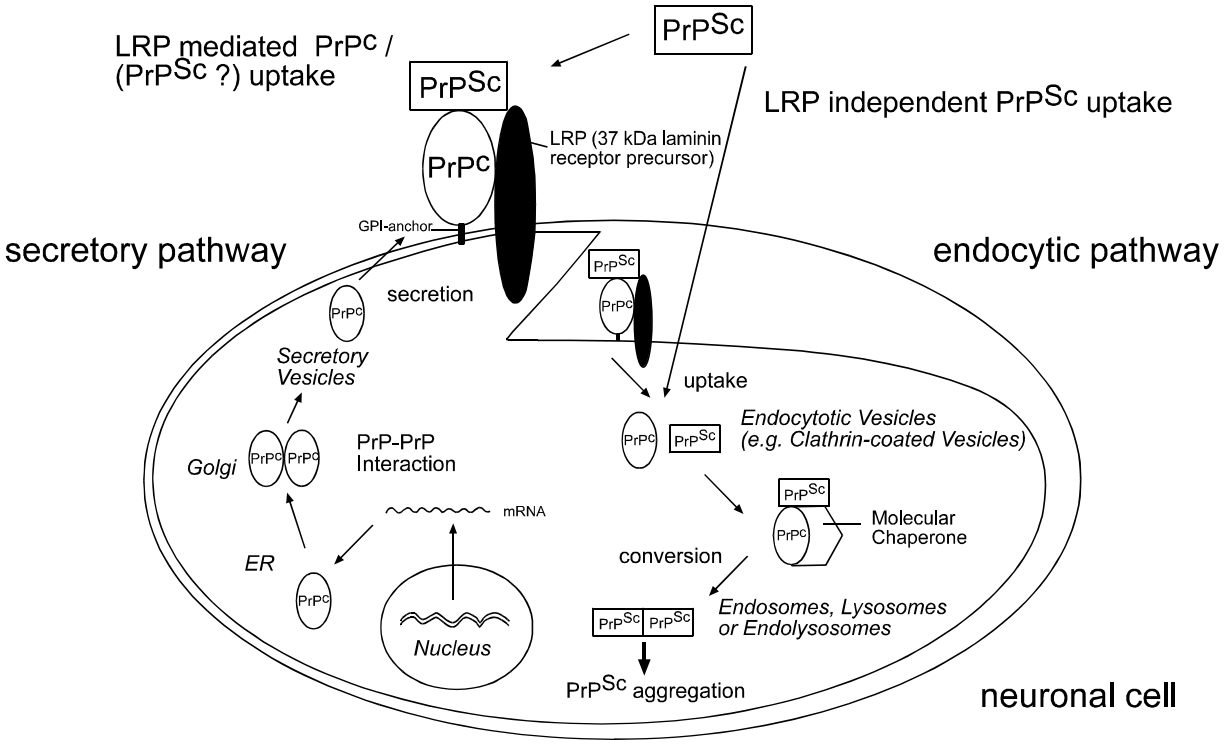

Fig. 3: Model of the life cycle of prions. PrP^c is synthesized in the rough endoplasmatic reticulum (ER), and after passing through the secretory pathway including the Golgi and secretory vesicles, reaches the surface of a PrP^{Sc} infectable cell where it is anchored via a glycosylphosphatidyl inositol (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.

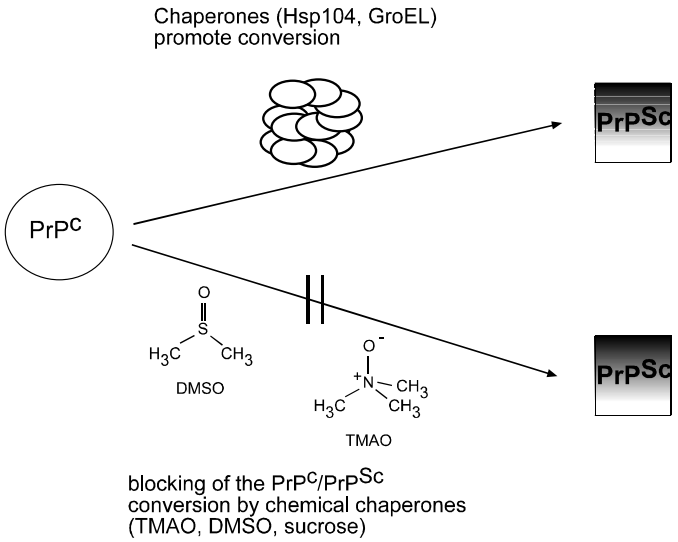

Fig. 4: Influence of molecular and chemical chaperones on the conversion process of PrP^{c} to PrP^{Sc}. Molecular chaperones such as Hsp104 and GroEL promote the conversion reaction whereas the chemical chaperones TMAO, DMSO and sucrose prevent PrP^{Sc} formation.

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.

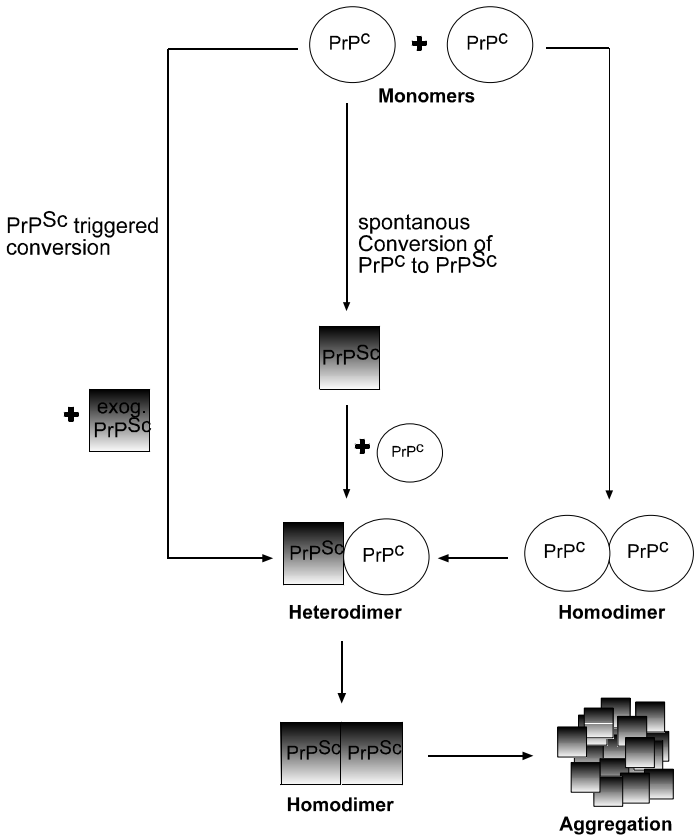

Fig. 5: Scheme of the conversion process of PrP^c to PrP^{Sc}. Three possibilities for the conversion of PrP^c into PrP^{Sc} do exist. An exogenous PrP^{Sc} triggers the conversion of PrP monomers leading directly to the hypothesized heterodimer consisiting of PrP^c and PrP^{Sc}. Genetic predisposition 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.

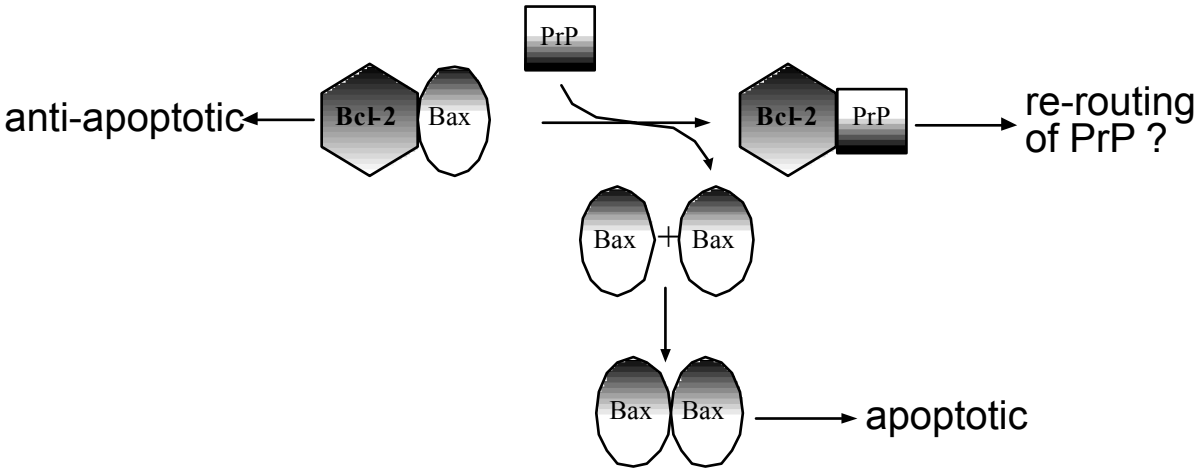

Fig. 6: Schematic view of Bcl-2::Bax, Bcl-2::PrP and Bax::Bax transitions and their possible role in cellular functions.

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.

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 deposition	Used for the treatment of malignancies	Tagliavini *et al*. (1997)
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)

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).

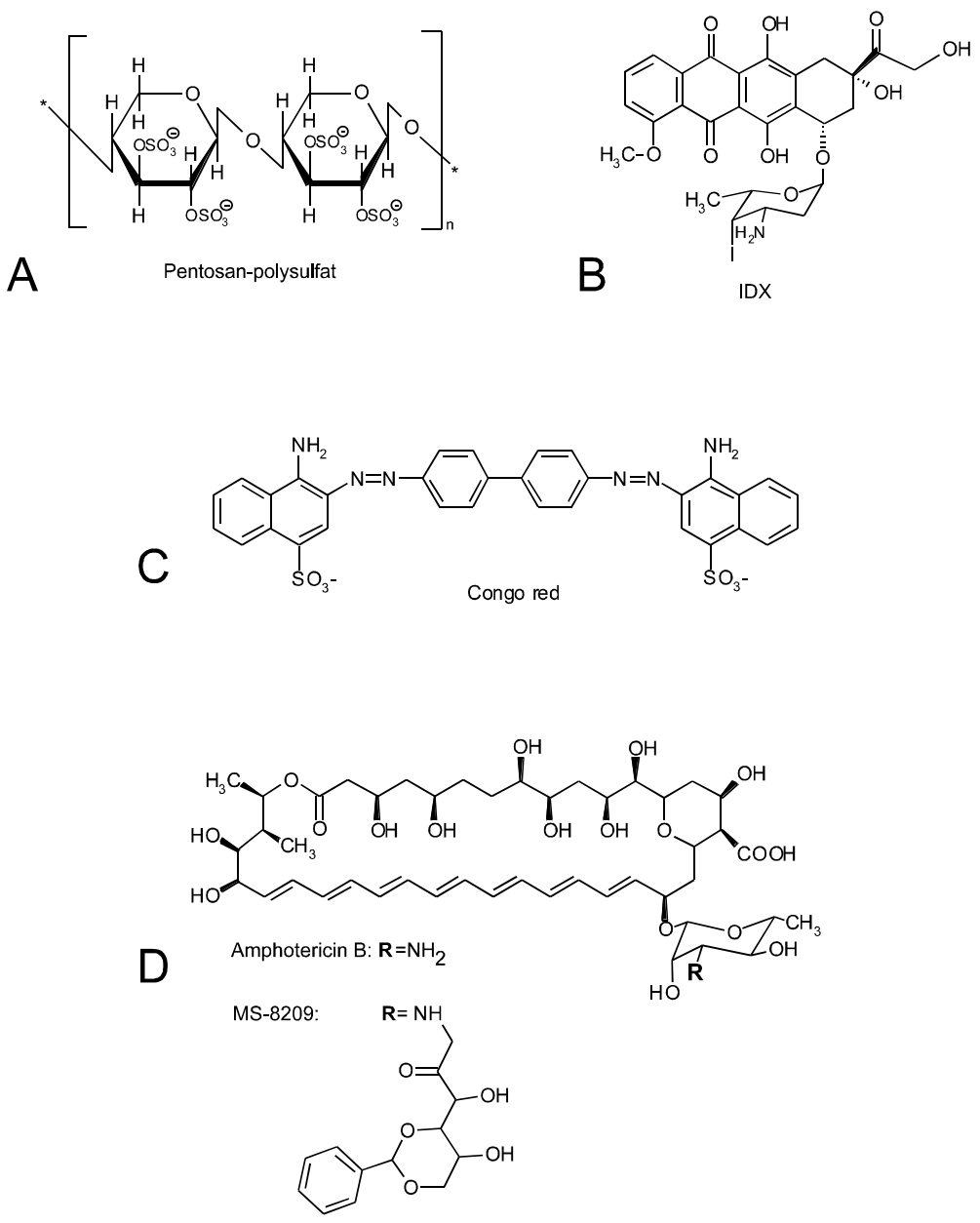

Fig. 7: Antiscrapie drugs of four different classes. (A) Pentosan polysulfate as a powerful drug belonging to the polyanion family. (B) IDX a derivative of doxorubicin. (C) Congo red belonging to the diazo dyes. (D) Amphotericin B and MS-8209 belonging to the family of polyene macrolide antibiotics.

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

The 37-kDa/67-kDa laminin receptor acts as the cell surface receptor for the cellular prion protein

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Abstract

Recently, we identified the 37-kDa laminin receptor precursor (LRP) as an interactor for the prion protein (PrP). Here, we show the presence of the 37-kDa LRP and its mature 67-kDa form termed high-affinity laminin receptor (LR) in plasma membrane fractions of N2a cells, whereas only the 37-kDa LRP was detected in baby hamster kidney (BHK) cells. PrP co-localizes with LRP/LR on the surface of N2a cells and Semliki Forest virus (SFV) RNA transfected BHK cells. Cell-binding assays reveal the LRP/LR-dependent binding of cellular PrP by neuronal and non-neuronal cells. Hyperexpression of LRP on the surface of BHK cells results in the binding of exogenous PrP. Cell binding is similar in $PrP^{+/+}$ and $PrP^{o/o}$ primary neurons, demonstrating that PrP does not act as a co-receptor of LRP/LR. LRP/LR-dependent internalization of PrP is blocked at 4°C. Secretion of an LRP mutant lacking the transmembrane domain (aa86 to aa101) from BHK cells abolishes PrP binding and internalization. Our results show that LRP/LR acts as the receptor for cellular PrP on the surface of mammalian cells.

Introduction

The prion protein is an ubiquitous host protein expressed by all known mammals (Oesch *et al*., 1991; Oesch *et al*., 1985; Schätzl *et al*., 1995) predominantly in the brain (Chesebro *et al*., 1985). While its exact function is still unknown, a role has been proposed in synaptic transmission by neuronal cells (Collinge *et al*., 1994; Fournier *et al.*, 1995; Kitamoto *et al.*, 1992), in sleep behaviour (Tobler *et al.*, 1996) and in cell survival (Kuwahara *et al.*, 1999) (for review see (Weissmann, 1996)). The Purkinje cell degeneration (Sakaguchi *et al.*, 1996), however, was not due to the lack of PrP, but to overexpression of doppel (Dpl) (Moore *et al.*, 1999). PrP binds copper *in vivo* (Brown *et al.*, 1997) and reveals signal transduction activity by activating tyrosine kinase Fyn (Mouillet-Richard, 2000). PrP is essential for the development of transmissible spongiform encephalopathies (TSEs) (Bueler *et al.*, 1993) also known as prion diseases, which represent fatal neurodegenerative diseases such as scrapie in sheep, BSE in cattle and Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans (for review see (Lasmézas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann and Aguzzi, 1997)).

It is thought that an abnormal form of PrP, termed PrPres for its partial resistance to proteolytic digestion, which accumulates in the brain of infected individuals, is a major component of the infectious agent of TSEs (Prusiner, 1982). The process leading to the

harmful form of the protein results in a conformational change of α-helices or unstructured regions of PrP^c to β-sheet structures in PrPres (Caughey *et al.*, 1991). It is still unknown if the neuronal death observed in TSEs is due to a loss of function of PrP^c or to the toxicity of PrPres. In this context, the identification of the cellular receptor for PrP would be a key step towards both, the understanding of disease pathogenesis and the development of therapeutics.

Within the life cycle of the prion protein, PrP^c is transported to the cell surface where it remains GPI anchored. PrP^c is internalized via clathrin coated pits (Shyng *et al.*, 1994) or caveolae like domains (Vey *et al.*, 1996). The conversion of PrP^c into PrPres may take place at the cell surface, in endosomes, lysosomes or endolysosomes. This 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 identified as an interactor for PrP^c (Edenhofer *et al.*, 1996). The presence of a specific so far unidentified cell-surface receptor for PrP has been deduced from complementary hydropathy (Martins *et al.*, 1997). Simultaneously, we identified the 37-kDa laminin receptor precursor (LRP) – which represents the precursor of 67-kDa laminin receptor (LR) - as an interactor for the prion protein in a yeast two-hybrid screen (Rieger *et al.*, 1997) and hypothesized that LRP could act as a receptor or co-receptor for PrP (for review (Gauczynski *et al.*, 2001; Rieger *et al.*, 1999)). In the present study, we confirm the presence of the 37-kDa LRP and its mature 67-kDa isoform at the plasma membrane of N2a cells. We found that PrP co-localizes with LRP/LR at the surface of N2a and with LRP on BHK cells, the latter hyperexpressing LRP and PrP by recombinant (rec.) Semliki Forest virus vectors (for review on the SFV-system see (Liljestrom and Garoff, 1991; Tubulekas *et al.*, 1997)). The relationship between 37-kDa LRP and 67-kDa LR is unknown so far (for review see (Gauczynski *et al.*, 2001)). As we observed in this study both forms of the receptor in plasma membrane fractions of N2a cells, we suppose that both forms may act as the receptor for cellular PrP. We investigated the role of LRP/LR as a receptor for cellular PrP by the development of various cell-binding/internalization assays for PrP. We further studied by PrP hyperexpression on baby hamster kidney (BHK) and Hela cells the possible role of endogeneous PrP acting as a co-receptor for LRP/LR on the cell surface. Employing an LRP deletion mutant lacking the transmembrane domain of LRP, termed LRPdelTMD, we also investigated the necessity of LRP for PrP binding and internalization. We conclude from these data that 37-kDa LRP/67-kDa LR acts as the main cell-surface receptor for PrP.

Results

Co-localization of 37-kDa LRP/67-kDa LR with PrP on the surface of neuroblastoma cells

Immunofluorescence (IF) analysis of non-permeabilized murine neuroblastoma cells (N2a[MHM2]) employing LRP- (Figure 1A) and PrP- (Figure 1B) specific antibodies demonstrated that PrP and LRP/LR co-localize on the surface of these cells (Figure 1C). The integrin LR VLA6 failed to co-localize with PrP (Figure 1D-F) and LRP/LR on the cell surface (Figure 1G-I). Fluorescence-activated cell (FAC) scans of non-permeabilized N2a cells employing an LRP specific antibody confirmed the cell-surface location of LRP/LR (Figure 1J). The β-galactoside lectin galectin-3 (gal-3) (Yang *et al.*, 1996), which was used as a control throughout the experiments because of a previously reported cross-reactivity with LRP (Buto *et al.*, 1998), is not expressed on the surface of N2a cells (Figure 1L). Western blot analysis of cytoplasm free plasma membrane fractions of N2a cells using a monoclonal antibody against LRP/LR revealed that the 37-kDa form (LRP) and to a lesser extent its mature 67-kDa form (LR), are located on the plasma membrane of N2a cells (Figure 1K). IF and FACscans of non-permeabilized primary cultures of mouse cortical neurons (data not shown) and HeLa cells (Figure 4B) also demonstrated the cell-surface location of LRP/LR on these cells, used for PrP binding experiments.

Location and orientation of LRP and human PrP on BHK cells transfected by recombinant Semliki Forest virus RNA

In order to investigate more precisely the localization and orientation of LRP on the surface of mammalian cells, the Semliki Forest virus (SFV) system was used to express rec. LRP::FLAG in BHK cells. Immunofluorescence analysis (Figure 2A, left inset) and FACscans (Figure 2D) reveal a low level of endogenous LRP expression. Gal-3 was not expressed on the surface of BHK cells (Figure 2F). Detection of LRP::FLAG at the surface of SFV LRP-FLAG RNA transfected BHK cells with a FLAG antibody (Figure 2A) demonstrates that LRP acts as a type 2 receptor with its C-terminus oriented to the extracellular space. Flow cytometry confirmed the cell-surface location of LRP::FLAG (Figure 2E). Endogeneous LRP (Figure 2G, lane 1), hyperexpressed LRP::FLAG (Figure 2G, lanes 2 and 4) and human PrP (Figure 2G, lane 6) are located at the plasma membrane of BHK cells. Expression of LRP::FLAG in this cell system did not result in the 67-kDa form of the LR. These data lead to the model for LRP depicted in Figure 2C showing the laminin-binding domain (Castronovo *et al.*, 1991) coinciding with the direct PrP-binding site located

between amino acids (aa)161 and 179 (Hundt *et al.*, 2001). An LRP mutant (LRPdelTMD) lacking the proposed transmembrane domain (Castronovo *et al.*, 1991) secreted to the extracellular space of BHK cells (Figure 5D) demonstrating that this region indeed represents the transmembrane domain of LRP. Transfection of SFV human PrP RNA into BHK cells led to the translocation of non-tagged human PrP to the surface of BHK cells (Figure 2B). We then aimed to verify whether the cellular location of LRP and PrP would allow them to interact with each other. Co-expression of LRP::FLAG and human PrP in BHK cells proved that LRP (Figure 2H) and PrP (Figure 2I) co-localize to a large extent on the cell surface (Figure 2J).

LRP/LR-dependent binding of human PrP to mammalian cells

To investigate a possible role of LRP/LR for the PrP binding and internalization, we established cell-binding assays with prion proteins. We confirmed that the PrP^c moiety of rec. GST::huPrP, employed in most of our assays, displays a conformation similar to native PrP^c by CD spectroscopy (Figure 3E) as shown previously for glutathione S-transferase (GST)-fused hamster PrP23-231 (Volkel *et al.*, 1998). The binding of GST::huPrP23-230 to N2a cells (Figure 3A) can be totally abolished by pre-incubating the cells with the LRP antibody W3 (Figure 3A, inset). Exogeneous PrP bound to the cell surface (Figure 3C) and co-localized partly with LRP/LR (Figure 3B and D). The binding curve deduced from western blot quantification of the GST::huPrP binding to N2a cells (Figure 3 F) reveals that at a GST::PrP^c concentration of 4 µg/ml (used in the co-localization assay) the receptor molecules were not saturated (visible as green dots on the cell surface in Figure 3 D). This is a possible explanation for the incomplete co-localization, which is compatible with the k_D of 1 x 10^{-7} mol/l deduced from this binding curve. At this GST::PrP^c amount there might also be more receptor molecules on the cell surface than could bind 4 µg of PrP. The association of rec. PrP with N2a cells was competed in a dose-dependent manner with the LRP antibody W3 (Figure 3G; Table I). Recently, a homology of the N-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. Antibodies directed against the molecular chaperones Hsp60, 70 or 90 (Figure 3H; Table I), however, did not influence the PrP-binding reaction. GST::huPrP23-230, saturated with a GST antibody prior to exposure, bound also to human NT2 cells (data not shown). Authentic PrP^c from hamster brain membrane preparations bound LRP/LR-dependent to MNB cells (Figure 3I). All experiments performed to verify the strict LRP/LR and PrP specificity of the binding reaction are summarized in Table I. Pre-immune serum,

antibodies directed against GST, GFAP, laminin or gal-3 revealed no effect. Antibodies against other LRs such as the lutheran protein (El Nemer *et al.*, 1998) and the integrin laminin receptor VLA6 (Magnifico *et al.*, 1996) did not inhibit the binding of PrP. In addition, we observed that the lutheran protein failed to interact with LRP in the yeast two hybrid system (data not shown). Saturation of the rec. protein with the PrP antibody JB007 led to a complete inhibition of the binding. A monoclonal antibody directed against aa 285-295 of LRP/LR failed to compete for the binding of GST::huPrP, whereas the monoclonal LRP/LR antibody directed against aa 167-243 reduced the binding of PrP to neuronal cells (Figure 3G and Table I).

Hyperexpression of LRP on the cell surface of BHK cells by the SFV system enhanced binding of recombinant PrP

Next we aimed to verify whether a quantitative relationship exists between PrP binding and the amount of LRP available on the cell surface. Untransfected BHK cells with a low level of endogenous LRP (Figure 3J, lower panel and Figure 2A, left inset) in the absence of any detectable LR bind only barely detectable amounts of rec. PrP (Figure 3J, upper panel and Figure 3L, triangles). In contrast, hyperexpression of LRP::FLAG at the surface of BHK cells (Figure 3J, lanes 1-5, lower panel and Figure 2A) led to an enhanced dose-dependent binding of GST::huPrP (Figure 3J, lanes 1-5, upper panel and Figure 3L, squares). Binding of non-, mono- and diglycosylated human PrP (without any tag) produced in the SFV-system was significantly increased when LRP::FLAG was hyperexpressed at the cell surface (Figure 3K, lanes 1-5 versus 6-10 and Figure 3M, squares versus triangles). Next, we wanted to verify whether additional PrP on the cell surface influences the binding of externally added rec. PrP. The co-expression of LRP::FLAG (Figure 3J, lanes 6-10, lower panel) and human PrP (Figure 3J, lanes 6-10, upper panel) on the cell surface reduced the dose-dependent GST::huPrP binding (Figure 3J, lanes 6-10, upper panel and Figure 3L, diamonds) when compared with cells transfected with LRP::FLAG only (Figure 3L, squares). This finding suggests that PrP does not act as a co-receptor for LRP for the binding of externally added PrP on the surface of mammalian cells.

Binding behaviour of PrP to HeLa cells hyperexpressing PrP at the cell surface

The function of PrP^{c} is unknown. However, due to its topography, it has been hypothesized that it could function as a receptor (Weissmann, 1996). We wanted to know whether PrP acts as a co-receptor for LRP/LR. To this purpose, we determined whether transiently transfected

HeLa cells (~ 10-20 % of total cells) with a low level of endogeneous PrP (Figure 4E, non-transfected cells) and a high level of LRP/LR on the cell surface (Figure 4B) hyperexpressing human PrP on their surface (fine red frame, Figure 4A) showed an enhanced binding of externally added rec. PrP compared with non-transfected HeLa cells. The binding of rec. PrP to cells hyperexpressing PrP was not increased compared with normal cells (Figure 4D-F, compare cells stained in red with the others). Both the binding of PrP to transfected and to non-transfected cells could be efficiently inhibited with the LRP specific antibody (data not shown).

Similar binding of PrP to neurons isolated from $PrP^{o/o}$ mice and PrP wild-type mice

In order to confirm that PrP at the cell surface does not participate in the binding of rec. PrP, we performed binding assays on primary cultures of neurons from $PrP^{o/o}$ mice versus wild-type mice. The binding of GST::huPrP was similar for both types of neurons (Figure 4G and I) and was completely abolished by pre-incubating $PrP^{+/+}$ or $PrP^{o/o}$ cells with the LRP antibody W3 (Figure 4H, J).

LRP/LR-dependent binding and internalization of recombinant PrP by N2a cells

The internalization of PrP was shown on N2a cells incubated with GST::huPrP and trypsinized (Figure 5A, lane 3); it was blocked by the LRP antibody (Figure 5A, lane 5), whereas the gal-3 antibody had no effect (Figure 5A, lane 4). Lowering the incubation temperature to 4°C resulted in a complete inhibition of the PrP internalization process (Figure 5B, lane 3) confirming that the process is active and receptor-mediated. These results demonstrate the LRP/LR-dependent internalization of the human prion protein.

Secretion of an LRP mutant lacking the transmembrane domain totally abolished PrP binding and internalization

In order to prove the necessity of LRP for the binding and internalization process, we compared the GST::huPrP binding/internalization by BHK cells expressing full-length LRP with cells expressing an LRP mutant lacking the proposed transmembrane domain (aa 86 to 101) (Castronovo *et al.*, 1991) termed LRPdelTMD. This mutant was detected in the supernatant of the cells and in the crude lysates revealing its presence at high amounts in the secretory pathway and its secretion to the extracellular space (Figure 5D, lanes 7-9, middle and lower panel, respectively), whereas full-length LRP::FLAG was detected in the crude lysate only (Figure 5D, lanes 4-6, middle panel). Binding and internalization of GST::huPrP

was observed in cells expressing wild-type LRP (Figure 5D, upper panel, lanes 5 and 6) but not in those expressing LRPdelTMD (Figure 5D, upper panel, lanes 8 and 9). Untransfected BHK cells having an extremely low level of endogeneous LRP bound no or only minimal amounts of externally added GST::huPrP (Figure 5D, lane 2). Binding and internalization of non-tagged highly glycosylated human PrP by LRP::FLAG hyperexpressing cells (Figure 5D, lanes 10 and 11) confirmed the observations made with GST-tagged human PrP. Levels of endogeneous LRP, as well as LRP::FLAG and LRPdelTMD::FLAG were only marginally reduced after trypsin treatment due to the fact that significant amounts of these proteins are located in the secretory pathway. Densitometric measurements revealed that N2a (Figure 5A and B) and BHK cells (Figure 5D) internalize between 25 and 50% of the bound PrP.

Discussion

The interaction of PrP^c with 37-kDa LRP suggested that LRP and its mature 67-kDa LR might act as a receptor or co-receptor for cellular PrP (Rieger *et al.*, 1997). In order to investigate this hypothesis we initiated a series of cell-binding/internalization assays employing neuronal and non-neuronal cells, recombinant as well as authentic prion proteins and a series of recombinant wild-type and mutated LRP molecules.

Localization of LRP/LR

A prerequiste for LRP/LR-dependent binding/internalization of PrP is the cell-surface location of LRP/LR. LRP has been found on 40S ribosomes and was dubbed p40 (Auth and Brawerman, 1992), in the nucleus (Sato *et al.*, 1996) and on the cell surface. 37-kDa LRP is located in plasma membrane fractions of mosquito cells acting as a receptor for the Venezuelan equine encephalitis virus (Ludwig *et al.*, 1996), in cell wall fractions of *Candida albicans* (Lopez-Ribot *et al.*, 1994) and on the cell surface of mammalian cells such as Madin-Darby canine kidney cells (Salas *et al.*, 1992). We showed by IF, flow cytometry and analysis of plasma membrane fractions that the 37-kDa LRP is located on the surface of neuroblastoma cells and non-transfected or LRP::FLAG hyperexpressing BHK cells. The 67-kDa form of the LR locates also to the cell surface (for review see (Gauczynski *et al.*, 2001)) where it acts as a receptor for the Sindbis virus (Wang *et al.*, 1992). We showed the presence of 67-kDa LR in plasma membrane fractions of N2a cells and concluded that the 37-kDa LRP/67-kDa LR might act as a receptor for PrP at the plasma membrane. The 37-kDa LRP/67-kDa LR polymorphism is unsolved so far. The association of cell-surface molecules

such as HSPGs with 37-kDa LRP might explain the appearance of the 67-kDa form of the receptor (Hundt *et al.*, 2001). LRP::FLAG hyperexpressing BHK cells revealed the cell-surface localization of LRP with its C-terminus oriented to the extracellular space enabling prion proteins to interact with PrP-binding domains on LRP. In summary, we showed (i) the membrane location of LRP/LR and (ii) the co-localization of PrP with LRP/LR on the surface of neuroblastoma cells and LRP/PrP hyperexpressing BHK cells.

LRP/LR-dependent binding of PrP to cells

For PrP binding and internalization experiments, we used externally added recombinant human PrP or authentic hamster prion protein, and a series of mammalian cells including murine neuroblastoma cells (N2a, MNB), primary cultures of neurons, human teratocarcinoma (NT2) and BHK cells. We proved LRP/LR-dependent binding of GST::huPrP and authentic hamster PrP to these cells. The k_D for the binding of rec. PrP to N2a cells was of $1x10^{-7}$ mol/l, which is in good agreement with the k_Ds of other cell-surface receptors such as the *N*-formyl peptide receptor (Christophe *et al.*, 2001) or the proteinaceous receptor on the surface of antigen presenting cells (Sondermann *et al.*, 2000).

The strict LRP/LR specificity of the PrP binding to NT2 and N2a cells was demonstrated in competition assays with a series of different antibodies (Table I). Whereas the LRP antibody W3 raised against the entire protein (Rieger *et al.*, 1997) competed totally for the binding of GST::huPrP to neuronal and non-neuronal cells, mAb LRP285-295 did not compete for the binding since aa 285-295 stretches outside the PrP binding domain (Hundt *et al.*, 2001). mAb LRP167-243 encompassing parts of the direct binding domain (Hundt *et al.*, 2001) was able to reduce the binding of PrP. Antibodies against the lutheran protein representing an erythroid receptor for laminin (El Nemer *et al.*, 1998), failed to compete for PrP cell binding. This receptor did not interact with PrP in the yeast two-hybrid system (data not shown). Anti-integrin receptor VLA6 antibodies (Magnifico *et al.*, 1996) and anti-β-galactoside lectin gal-3 antibodies also failed to compete for the PrP-binding reaction. VLA6 does not co-localize with PrP and LRP/LR on the cell surface. The use of LRP hyperexpressing BHK cells demonstrated the quantitative relationship between the number of LRP receptor molecules and the PrP-binding process.

LRP/LR-dependent internalization of PrP

N2a cells internalized 25-50% of the human PrP bound to the cell surface in an LRP/LR-dependent manner. The PrP internalization process represents an active receptor-mediated

event, confirmed by lowering the incubation temperature of N2a cells to 4°C resulting in a total blockage of PrP internalization without affecting PrP binding.

Expression of an LRP mutant lacking the putative transmembrane domain (LRPdelTMD) (Castronovo *et al.*, 1991) in BHK cells resulted in secretion of LRPdelTMD to the extracellular space confirming the hitherto indirect evidence that the transmembrane region stretches from aa 86 to 101. In contrast to full-length LRP hyperexpressing BHK cells, LRPdelTMD hyperexpressing cells did not bind or internalize PrP due to the secretion of the mutant to the extracellular space. Untransfected BHK cells similarly failed to bind and internalize PrP due to insufficient amounts of LRP on the cell surface, confirming that LRP is essential for PrP binding and internalization.

Endogeneous PrP does not act as a co-receptor for LRP/LR

The co-localization of LRP/LR and PrP at the surface of mammalian cells raises the possibility that PrP could act as a co-receptor for LRP/LR. Binding of rec. PrP to HeLa or BHK cells expressing additional PrP on the cell surface was not increased. On PrP plus LRP hyperexpressing BHK cells, PrP had even the adverse effect of hampering the increased binding due to LRP hyperexpression, probably by recruiting a proportion of the latter receptor for its own metabolism. Unaltered binding of rec. PrP to primary cortical neurons isolated from PrP knock-out mice confirmed that the absence of PrP on the cell surface had no influence on the LRP/LR-dependent PrP binding, demonstrating that endogeneous PrP does not act as a co-receptor for LRP/LR.

Role of LRP/LR in the metabolism of PrP and implications for the pathogenesis of TSEs

Our study has several implications in terms of both the metabolism of PrP^c and the pathogenesis of TSEs. Our co-localization and internalization data suggest that LRP/LR is essential for the normal cell cycle of PrP by mediating the internalization of PrP^c after its exposure at the cell surface. Internalization of PrP might occur via caveolae-like domains (Vey *et al.*, 1996) or via clathrin-coated pits (Shyng *et al.*, 1994). The receptor-mediated endocytosis of the protein (by LRP/LR), would direct the complex into clathrin-coated pits (for reviews see (Pley and Parham, 1993; Schmid, 1997)) rather than caveolae-like domains (for review (Maxfield and Mayor, 1997)). The role of LRP/LR as a receptor for the extracellular-matrix proteins laminin and elastin also suggests that its interaction with PrP may induce a signal involved in cell survival. In this respect, it has been shown that primary neurons devoid of PrP are more prone to neuronal death than their PrP expressing

counterparts (Kuwahara *et al.*, 1999). One possibility is that the interaction of an LRP/LR receptor on one cell with a PrP molecule on another cell would contribute to cell-to-cell communication essential for cell survival. Recently, a signal transduction activity of PrP by activating tyrosine kinase Fyn was described (Mouillet-Richard, 2000). The plasma membrane-associated LRP/LR (Figure 1 and 2) might mediate the signal transduction of the extracellular GPI-anchored PrP with the intracellular plasma membrane-associated Fyn kinase involving cell-surface HSPGs (Hundt *et al.*, 2001).

The fact that PrP^c binds to and is internalized by LRP/LR raises the possibility that PrPres is also bound/internalized by LRP/LR. The expression of LRP/LR in human small intestinal mucosa (Shmakov *et al.*, 2000) suggests that it may represent the portal of entry for PrPres after oral contamination. Our recent finding that LRP levels are increased in only those organs of rodents that accumulate PrPres, indicates that PrPres intervenes in the metabolism of LRP (Rieger *et al.*, 1997). Whether the internalization of PrPres relies on the presence of LRP/LR, PrP^c or both may be answered by cell biological studies. The generation of transgenic mice devoid of LRP/LR might also help to determine whether LRP/LR acts as the receptor for the infectious agent.

Also of relevance for pathogenesis, a saturation of the binding sites of LRP/LR may occur as a consequence of PrP accumulation in TSEs rendering the receptor unavailable to its ligand laminin and contributing to neurodegenerative processes. The absence of laminin-binding to its receptor sensitizes neurons to death, as demonstrated in mice affected with the weaver syndrome (Murtomaki *et al.*, 1995). Keeping in mind that laminin plays a central role in cell growth, differentiation and migration and that any interference with these functions may be deleterious for the organism, our findings demonstrating that PrP associates with and is internalized by LRP/LR into the cell open new avenues of research for anti-TSE therapeutics, either to block the entry of the infectious particle, to modify the metabolism of PrP or to interfere with the neurodegenerative process.

Materials and methods

Semliki Forest virus system

pSFV1-LRP::FLAG, pSFV1-LRPdelTMD::FLAG, pSFV1-huPrP1-253 were constructed as described in the Supplementary data (see pages 84-90). pSFV-1 (Liljestrom and Garoff, 1991), pSFV3-lacZ (Life Technologies) and the ORF from human PrP (Krasemann *et al.*,

1996) were used. Transfections of BHK-21 C13 cells with rec. SFV-RNAs (transfection efficiencies = 90-100 %) are described (see Supplementary data).

HeLa cells expressing huPrP

Human epitheloid carcinoma of cervix HeLa cells (ATCC CCL2) were transfected with *pCR3-uni*™-huPrP1-253 containing human *Prn*-p cDNA (Jaegly *et al.*, 1998) for huPrP expression as described (see Supplementary data).

Tissue culture of N2a, N2a [MHM2], MNB, NT2, HeLa, BHK, Sf9 cells, primary mouse cortical neurons, $PrP^{o/o}$ neuronal cultures

N2a, N2a [MHM2], MNB, NT2, HeLa, BHK, Sf9 cells, primary mouse cortical cultures and $PrP^{0/0}$ neuronal cultures (C. Weissmann, Zürich) were cultivated and prepared as described (see Supplementary data).

Generation of recombinant and authentic proteins

pAcSecG2T-huPrP was generated and rec. baculoviruses produced as described (see Supplementary data). Rec. GST, GST::huPrP23-230, dialyzed against 20 mM HEPES, pH 7.4 were expressed in the baculovirus system as described for GST::haPrP proteins (Weiss *et al.*, 1995; Weiss *et al.*, 1996). Authentic PrP^{c} was prepared from hamster brain membrane fractions (Meyer *et al.*, 1986). Human PrP was expressed in the SFV-system (see Supplementary data).

Far-UV Circular dichroism analysis

CD spectra of GST::huPrP23-230 were recorded as described (see Supplementary data).

PrP binding/internalization assays followed by immunofluorescence analysis, confocal microscopy or western blotting

For competition studies the cells were either pre-incubated with the individual antibody before the addition of rec. protein or the rec. protein was pre-incubated with the individual antibody before addition to the cells (inoculum saturation). After 18 h of incubation, cells were processed (with or without trypsin treatment) for IF-staining, confocal microscopy or western blotting as described in the see Supplementary data.

FACS analysis (flow cytometry)

Single-cell suspensions were prepared, cells treated and data acquisition obtained as described (see Supplementary data).

Isolation of plasma membranes

Plasma membrane preparations were done according to (Vleurick *et al.*, 1999).

Calculation of binding curves for recombinant PrP to cells and determination of the k_D for the interaction of PrP with LRP/LR

Calculations (NIH-Image)/k_D determination (Prism 3) were performed as described (see Supplementary data).

Antibodies

The antibodies used are described in the Supplementary data. For saturation of pAb LRP W3 with rec. PrP, immobilized GST::LRP was incubated with pAb LRP W3 and the supernatant assayed by IF on N2a/BHK cells.

Supplementary data

Supplementary data for this chapter are shown on pages 84 to 90.

Table I. Summary of the displacement capacity of antibodies for the binding of human PrP to neuronal cells.

Inoculum saturation[a]	Pre-incubation of cells[a]	Dilution	Binding Inhibition
pAb anti-PrP (JB007)			+++
Pre-immune serum (PrP immunization)			-
pAb/mAb anti-GST			-
	pAb anti-LRP	1/50 1/100 1/500 1/1000 1/10000	+++ ++ + - -
	pre-immune serum (LRP immunization)	1/50	-
	mAb anti-LRP 167-243	1/5 1/50	++ +
	mAb anti-LRP 285-295	1/5 1/50	- -
	pAb anti-GFAP	1/50	-
	pAb anti-laminin	1/50	-
	mAb anti-VLA6	1/50	-
	mAb anti-lutheran protein	1/50	-
	pAb anti-galectin-3 Ab	1/50	-
	mAb anti-Hsp60	1/50	-
	mAb anti-Hsp70	1/50	-
	mAb anti-Hsp90	1/50	-

[a]N2a and NT2 cells have been incubated with GST::huPrP23-231 after pre-incubation of the protein with the indicated antibodies (inoculum saturation) or after pre-incubation of the cells with the indicated antibodies. Antibody displacement capacities were analysed by Immunofluorescence. Concentrations of all undiluted antibodies used range between 1.5 and 1.7 mg/ml.

+++, ++, + : Inhibition of binding;

- : No inhibition of binding.

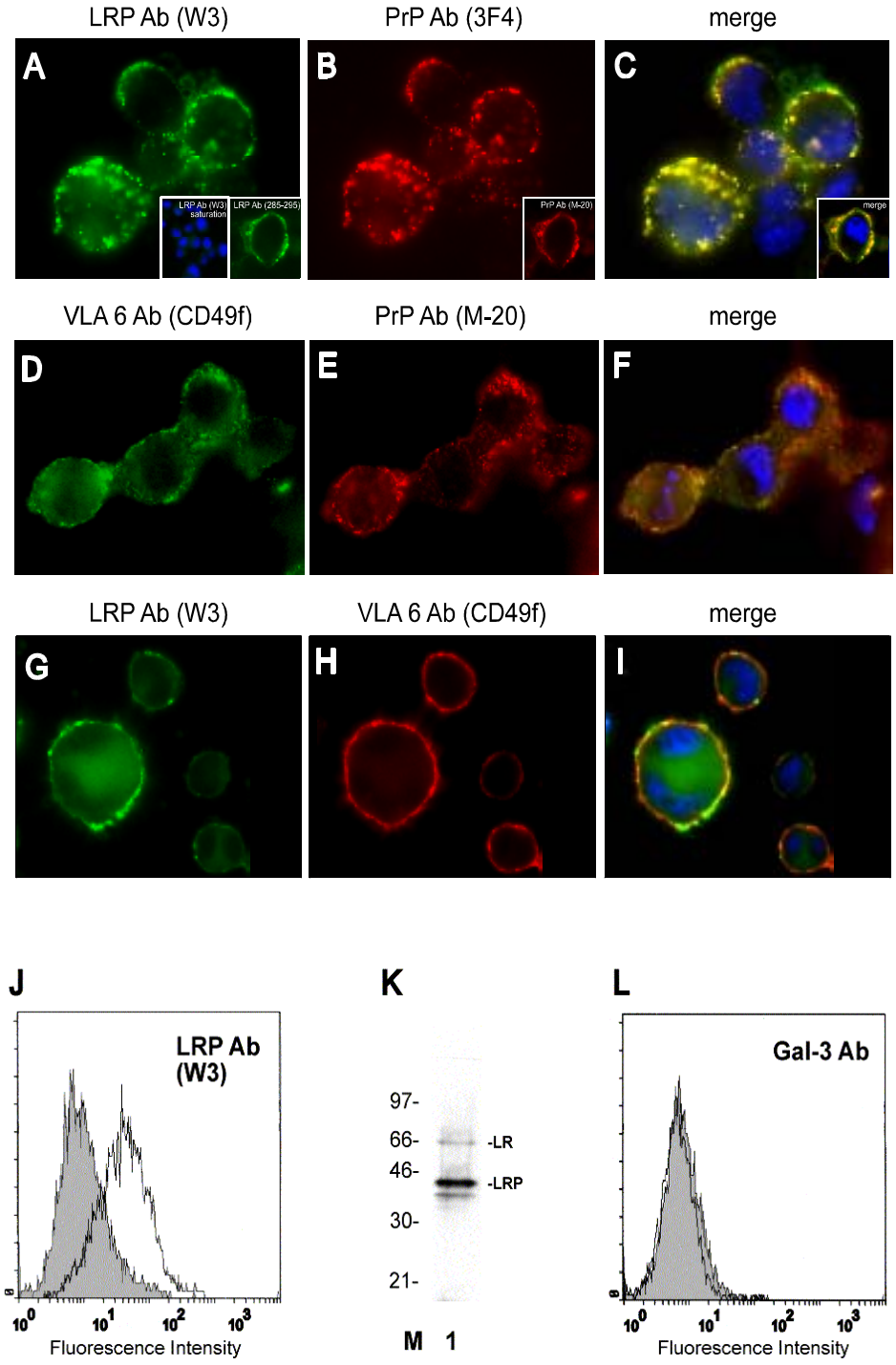


Fig. 1. Plasmamembrane-associated LRP/LR and PrP co-localize on the surface of neuroblastoma cells. Non-permeabilized N2a [MHM2] cells were incubated with the pAb LRP W3 [sec. Ab fluorescein isothiocyanate (FITC)] (**A**), pAb LRP W3 saturated with rec. GST::LRP [sec. Ab carbocyanine Cy2, 4′-6-diamidine-2-phenylindole (DAPI)] (**A**, left inset) or the mAb LRP (aa 285-295 of LRP, sec. Ab Cy2) (**A**, right inset) and the mAb PrP 3F4 [sec. Ab indocarbocyanine (Cy3)] (**B**), or the pAb PrP M-20 (**B**, inset). Merge of (A) and (B) DAPI staining (**C**) (magnification x630). N2a [MHM2] cells were incubated with the mAb VLA6 CD49f (sec. Ab Cy2) (**D**) and the pAb M-20 (sec. Ab Cy3) (**E**). Merge of (D) and (E) DAPI staining (**F**). N2a [MHM2] cells were incubated with the pAb LRP W3 (sec. Ab Cy2) (**G**) and the mAb VLA6 CD49f (sec. Ab Cy3) (**H**). Merge of (G) and (H) DAPI staining (**I**).

(**J**) Non-permeabilized N2a cells were analysed by FACscans. Filled profile, isotype control. non-filled profile, pAb LRP W3. Fluorescence intensity (abscissa) is plotted against relative cell numbers (ordinate). (**K**) Purified plasma membranes from N2a cells were analysed by western blotting employing a mAb LRP (directed against aa 167-243) (lane 1). Molecular weight markers are indicated. (**L**) Non-permeabilized N2a cells were analysed by FACscans. Filled profile, isotype control, non-filled profile, anti-gal-3 antibody. Fluorescence intensity (abscissa) is plotted against relative cell numbers (ordinate).

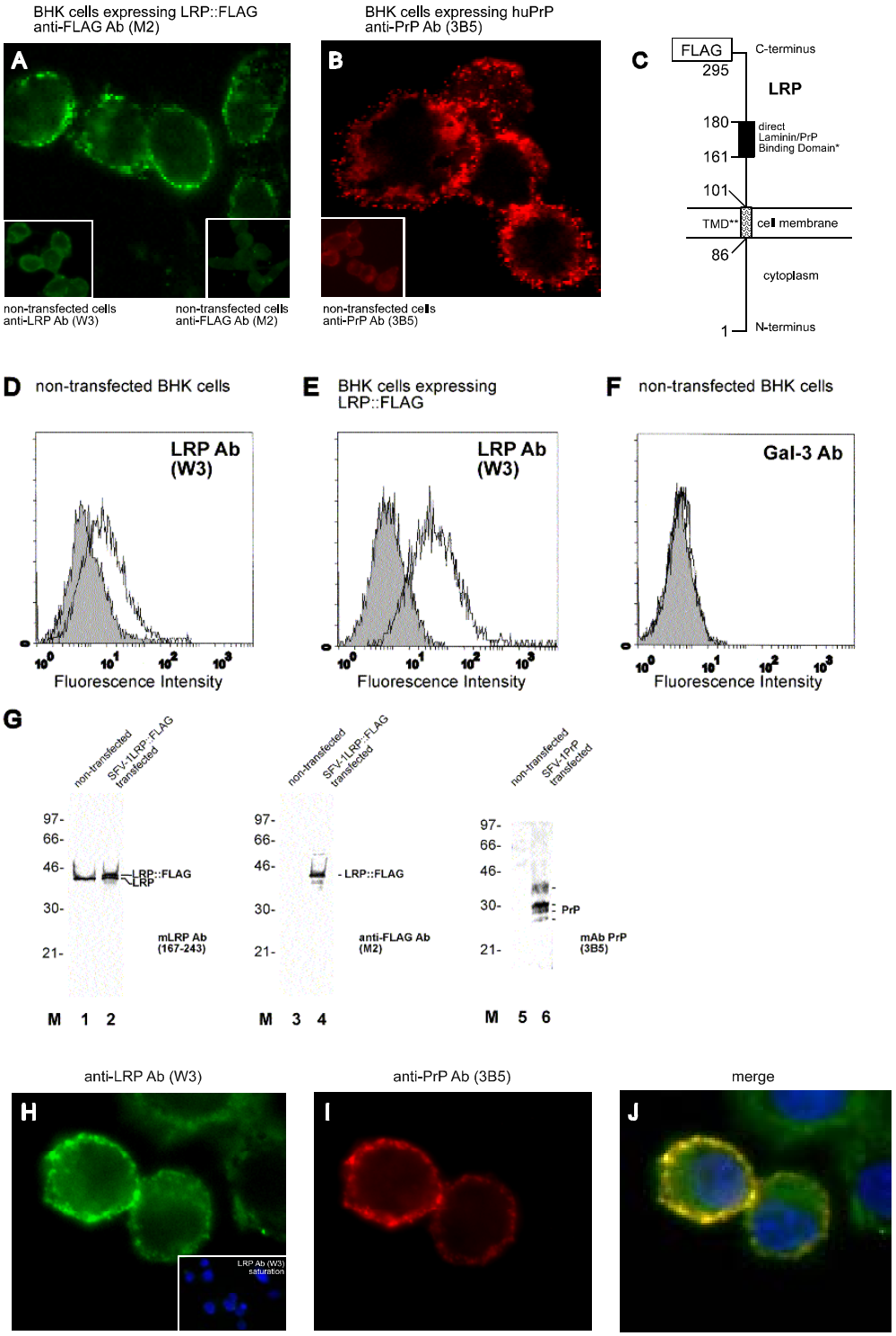
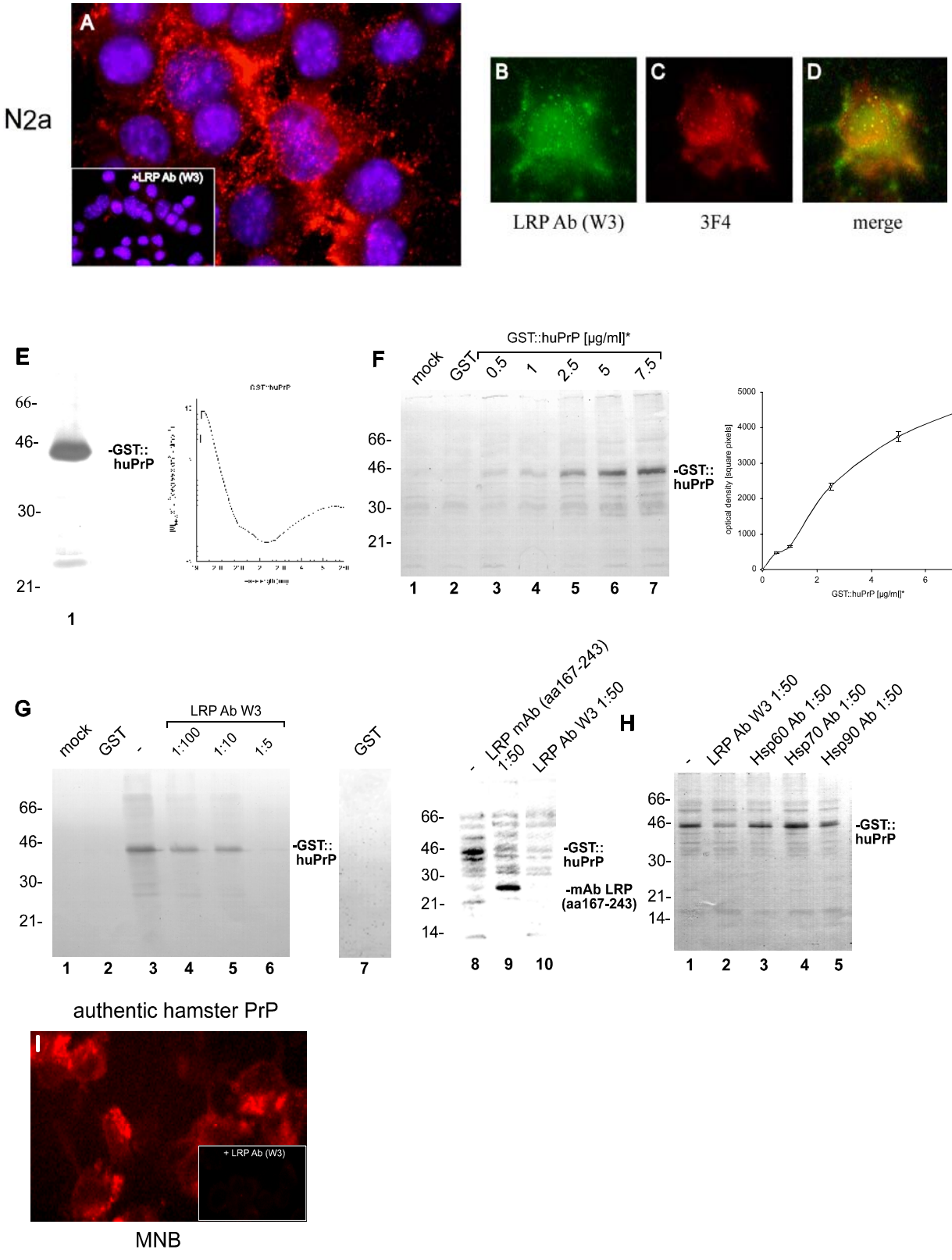


Fig. 2. Orientation, localization of LRP::FLAG and PrP and co-localization of both proteins in BHK cells transfected with rec. SFV RNAs. (**A**) Immunolocalization of LRP::FLAG to the cell membrane of non-permeabilized BHK cells transfected with rec. SFV LRP-FLAG RNA. Subcellular location was determined by IF using the mAb FLAG M2 (sec. Ab FITC). (Insets) Untransfected BHK cells incubated with the pAb LRP W3 (left), mAb FLAG M2 (right). (**B**) Immunolocalization of human PrP^{c} to the cell membrane of non-permeabilized BHK cells transfected with rec. SFV huPrP1-253 RNA. Subcellular location was determined by IF using the mAb PrP 3B5 (sec. Ab Texas Red). (Inset) Untransfected BHK cells (Ab 3B5). (**C**) Orientation of LRP on the cell surface. Orientation and localization of LRP on the cell surface is confirmed in (A). *The direct PrP binding domain suggested by (Rieger et al., 1997) and mapped in detail by (Hundt et al., 2001) is identical with the laminin-binding domain (Castronovo et al., 1991). **The transmembrane domain (TMD) was first suggested by (Castronovo et al., 1991). Secretion of an LRP mutant lacking the transmembrane domain (LRPdelTMD) to the extracellular space of BHK cells (Figure 5D) confirmed that the TMD indeed stretches from aa 86 to 101 of LRP. FACscans of non-permeabilized non transfected (**D**) and SFV LRP-FLAG RNA transfected BHK cells (**E**). Filled profile, isotype control; non-filled profile, pAb LRP W3. (**F**) FACscans of non-permeabilized non-transfected BHK cells. Filled profile, isotype control; non-filled profile, pAb gal-3 . Fluorescence intensity (abscissa) plotted against relative cell numbers (ordinate). (**G**) Western blot analysis of plasma membrane fractions from non-transfected and rec. SFV transfected BHK cells. Purified plasma membranes from non-transfected cells (lanes 1, 3 and 5) and cells transfected with SFV LRP-FLAG RNA (lanes 2, 4) or SFV huPrP1-253 RNA (lane 6) were analysed by western blotting using mAb LRP (aa 167-243) (lanes 1 and 2), mAb FLAG M2 (lanes 3 and 4) or mAb 3B5 (lanes 5 and 6). (H-J) IF-analysis of non-permeabilized BHK cells co-transfected with rec. SFV RNAs encoding for LRP::FLAG and human PrP. Immunostaining was performed using (**H**) the pAb LRP W3 non-sarurated and (**H**, inset) saturated with rec. GST::LRP (sec. Ab Cy2, DAPI staining) and (**I**) mAb 3B5 (sec. Ab Cy3). (**J**) Merge of (H) and (I) DAPI staining (magnification A, B, H-J, x630).


N2a
A
+LRP Ab (W3)
B
C
D
LRP Ab (W3)
3F4
merge
E
66-
46-
30-
21-
-GST::
huPrP
1
F
GST::huPrP [µg/ml]*
mock
GST
0.5
1
2.5
5
7.5
66-
46-
30-
21-
-GST::
huPrP
1 2 3 4 5 6 7
optical density [square pixels]
GST::huPrP [µg/ml]*
G
LRP Ab W3
mock
GST
-
1:100
1:10
1:5
GST
66-
46-
30-
21-
-GST::
huPrP
1 2 3 4 5 6
7
authentic hamster PrP
LRP mAb (aa167-243) 1:50
LRP Ab W3 1:50
-
H
66-
46-
30-
21-
14-
-GST::
huPrP
-mAb LRP
(aa167-243)
8 9 10
LRP Ab W3 1:50
Hsp60 Ab 1:50
Hsp70 Ab 1:50
Hsp90 Ab 1:50
66-
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-GST::
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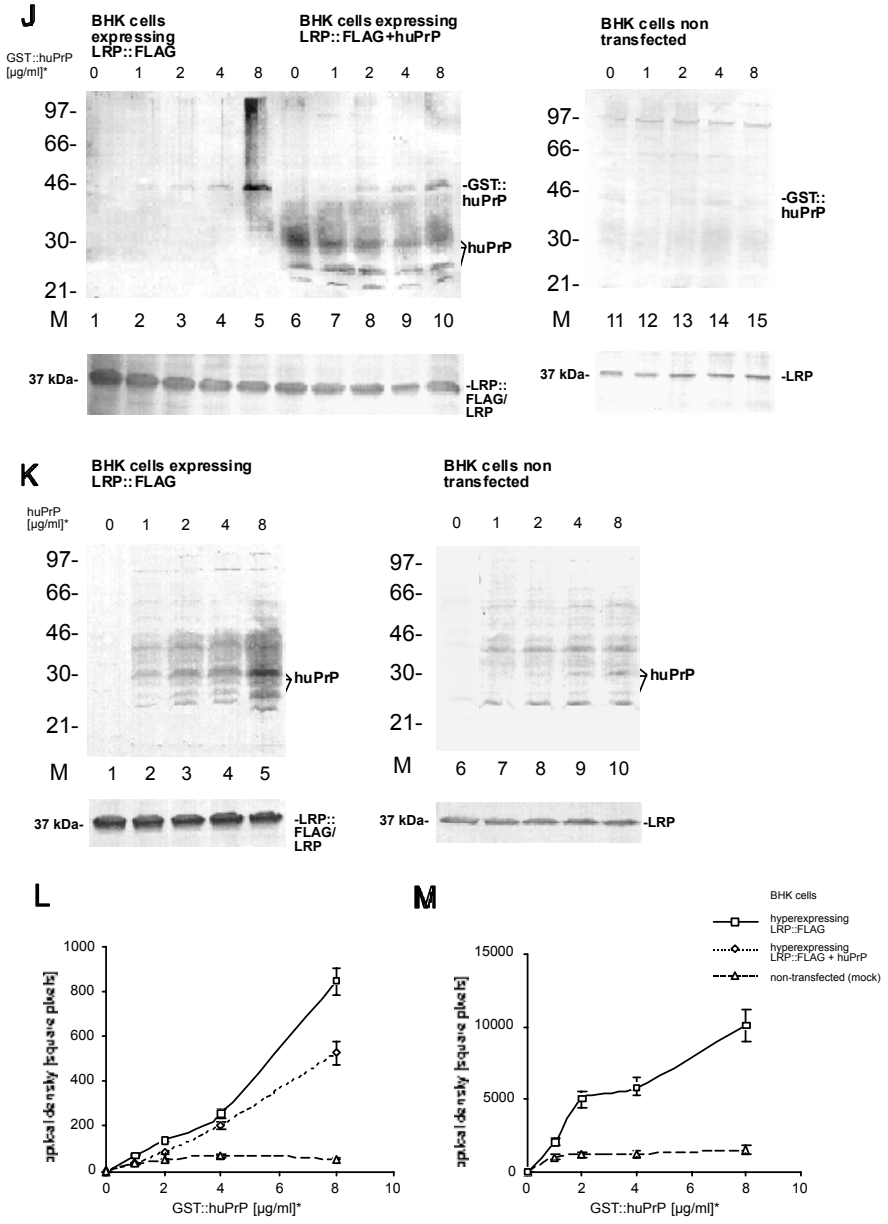

Fig. 3. LRP/LR-dependent binding of PrP by neuronal and BHK cells transfected with rec. SFV RNAs. (**A**) N2a cells (pre-incubated with pre-immune serum) were incubated with GST::huPrP23-230 (6 µg/ml). Binding of the rec. protein was assessed by IF using mAb GST (sec. Ab Cy3). (Inset) Preincubation of cells with pAb LRP W3 (dilution 1:50). (B-D) Co-localization of exogeneous GST::PrP23-230 with endogeneous LRP/LR on non-permeabilized N2a cells. Cells were incubated with GST::huPrP23-230 (4 µg/ml). Endogeneous LRP was detected by IF using the pAb LRP W3 (sec. Ab Cy2, **B**), exogeneous GST::huPrP was detected by mAb 3F4 (sec. Ab Cy3, **C**). Merge (**D**) of (B) and (C) (magnification A-D x 630). (**E**) Analysis of GST::huPrP23-230 by SDS-PAGE and FAR-UV CD spectroscopy. One microgram of GST::huPrP23-230 (lane 1) was analysed on a 12 %SDS-PA-gel stained with silver. FAR-UV CD spectrum (right panel) of GST::huPrP23-230 in 10 mM sodium phosphate buffer, pH 7.4. (**F**) Western blot analysis of the binding assay illustrated in (A-D). Binding of GST::huPrP to N2a cells. 500 ng/ml (lane 3), 1 µg/ml (lane 4), 2.5 µg/ml (lane 5), 5 µg/ml (lane 6) and 7.5 µg/ml (lane 7) of GST::huPrP23-230, 7.5 µg/ml GST (lane 2) and no protein (lane 1) were incubated with N2a cells. Total cell extracts were loaded. Protein detection by mAb 3F4 . The binding curve (right panel) of GST::huPrP23-230 to N2a cells was obtained by densitometric quantification (square pixels)

of the western blot signals for GST::huPrP23-230 plotted against the dose of rec. PrP (µg/ml). k_D = 1x10^{-7} mol/l (calculation described in Supplementary data). (**G**) pAb LRP W3 and mAb LRP (aa 167-243) displacement of the GST::huPrP binding to N2a cells. Cells were incubated in the absence of protein (lane 1), with 7.5 µg/ml GST (lane 2 and 7), 3 µg/ml GST::huPrP23-230 (lanes 3 and 8), 3 µg/ml GST::huPrP23-230 after pre-incubation with pAb LRP W3 at 1:100 (lane 4), 1:10 (lane 5), 1:5 (lane 6), mAB LRP (aa 167-243) at 1:50 (lane 9) and pAb LRP W3 at 1:50 (lane 10). Proteins were detected by mAb 3 F4 (lanes 1-6 and lanes 8-10) or the pAb GST (lane 7). (**H**) GST::huPrP displacement on N2a cells with antibodies directed against molecular chaperones. Cells were incubated with 3 µg/ml of GST::huPrP23-230 without antibodies (lane 1) and with pAb LRP W3 (lane 2), antibodies directed against Hsp60 (lane 3), Hsp70 (lane 4) and Hsp90 (lane 5). Antibody dilution: 1:50. Blots were developed with the mAb 3F4. (**I**) LRP-dependent binding of authentic PrP isolated from hamster brains on MNB cells. MNB cells were incubated with 2 µg/ml of purified PrP^c from hamster brain. Immunostaining was performed with the mAb 3F4 (sec. Ab Texas Red). (Inset) MNB cells saturated with the pAb LRP W3 (dilution 1:50) prior to PrP treatment (magnification x630). (J-M) Increased PrP binding by rec. SFV RNA transfected BHK cells overexpressing LRP at the cell surface. BHK cells (**J**) were either transfected with SFV LRP-FLAG RNA (lanes 1-5), SFV LRP-FLAG RNA plus SFV huPrP1-253 RNA (lanes 6-10) or non transfected (lanes 11 -15). Amounts of 0 µg/ml (lanes 1, 6 and 11), 1 µg/ml (lanes 2, 7 and 12), 2 µg/ml (lanes 3, 8 and 13), 4 µg/ml (lanes 4, 9 and 14) and 8 µg/ml (lanes 5, 10 and 15) of GST::huPrP23-230 were added to the cells. Total cell extracts were analysed by western blotting employing the mAb 3F4 (J, upper panels) or the pAb LRP W3 (J, lower panels). Please note that endogeneously expressed huPrP appeared as non-, mono- and diglycosylated isoforms (J). BHK cells (**K**) were transfected with SFV LRP-FLAG RNA (lanes 1-5) or non transfected (lanes 6-10). Amounts of 0 µg/ml (lanes 1 and 6), 1 µg/ml (lanes 2 and 7), 2 µg/ml (lanes 3 and 8), 4 µg/ml (lanes 4 and 9) and 8 µg/ml of huPrP23-230 (SFV system) (lanes 5 and 10) were added to the cells. Total cell extracts were analysed by western blotting with the mAb 3F4 (**K**, upper panels) and the pAb LRP W3 (**K**, lower panels). Please note that externally added rec. non-tagged human PrP used for binding studies appeared as non-, mono- and diglycosylated isoforms (panel K). Binding curves were obtained by quantitating the western blot signals for GST::huPrP in J (**L**) and for huPrP in K (**M**) by densitometry (square pixels). For binding studies the cells were incubated for 18 h with GST::huPrP before staining with the indicated individual antibody was performed. *GST::huPrP and huPrP concentrations represent the concentration of added recombinant protein in the cell media. Values (F, right panel, L and M) were calculated by optical scanning methods (see Supplementary data).

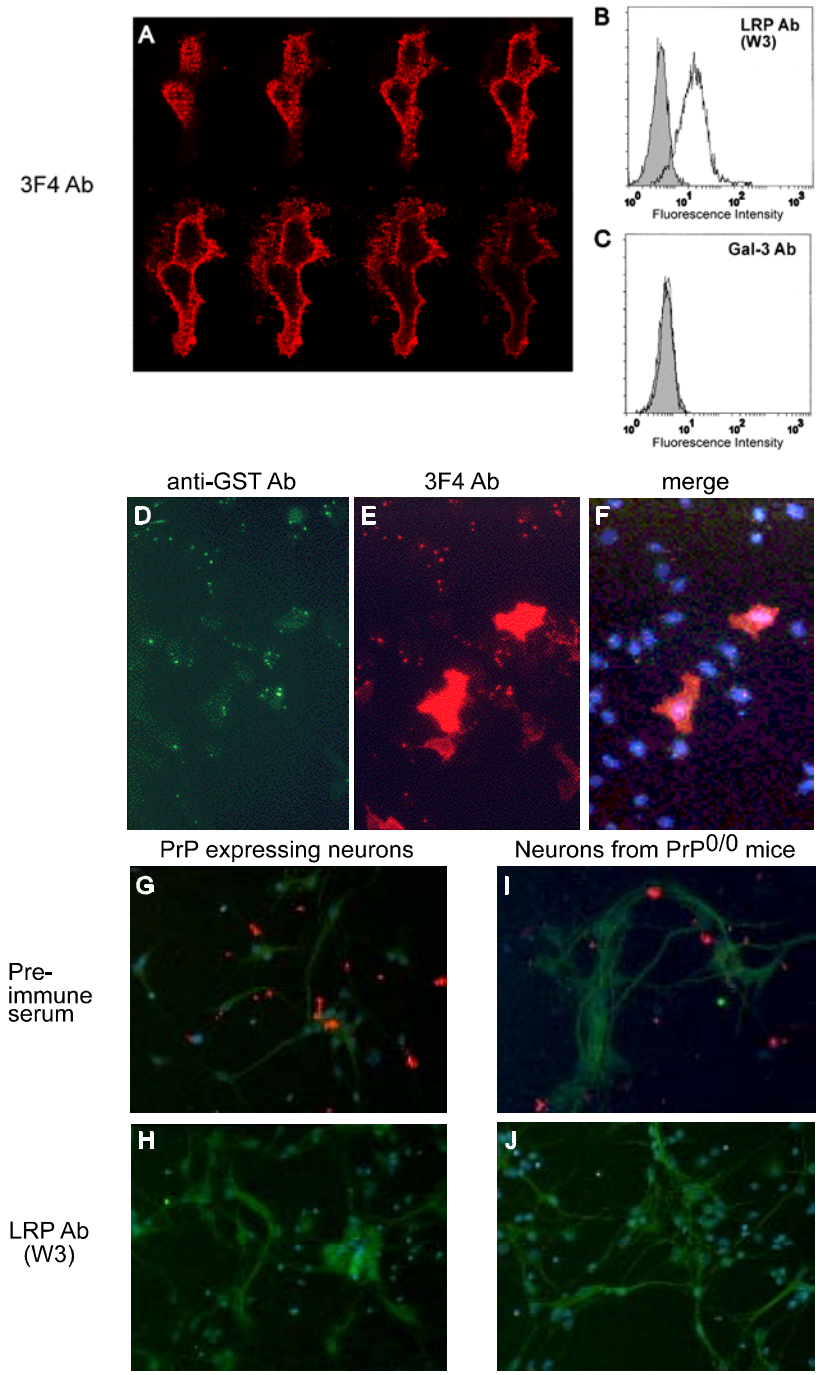

Fig. 4. Endogenous PrP does not act as a co-receptor of LRP/LR for the binding of exogeneous PrP. (A-F) Unaltered GST::huPrP23-230-binding by HeLa cells overexpressing human PrP at the cell surface. (**A**) Confocal z series of HeLa cells transiently transfected with cDNA encoding for human PrP1-253. Transfected HeLa cells were analysed employing the mAb 3F4 (sec. Ab Texas Red). Confocal scanning was performed from the cell surface (top panel left) towards the interior of the cell (bottom panel, right) (magnification x630). Non-permeabilized HeLa cells were analysed by FACscans. Filled profile, isotype control (**B** and **C**), non-filled profile, pAb LRP W3 (B), pAb gal-3 (C). Fluorescence intensity (abscissa) is plotted against relative cell numbers (ordinate). (D-F) Binding of GST::huPrP23-230 by HeLa cells transfected with pCR3-uni™-huPrP1-253. (**D**) Cells were analysed by IF with pAb GST (sec. Ab FITC), (**E**) Immunostaining with mAb 3F4 (sec. Ab Texas Red), (**F**) Triple labelling

with PrP and GST antibodies, DAPI staining. pCR3-uni™-huPrP1-253 transfected cells are red-colored (magnification x400). (G-J) LRP-dependent binding of GST::huPrP23-230 by primary culture of neurons isolated from PrP wild-type and $PrP^{o/o}$ mice. Primary cultures of neurons from wild-type mice (**G** and **H**) or $PrP^{o/o}$ mice (**I** and **J**) were incubated with GST::huPrP23-230 (4 µg/ml) after preincubation with either pre-immune serum (G and I) or pAb LRP W3 (dilution 1:50) (H and J). Immunostaining was performed with mAb 3F4, DAPI staining and neuron staining with MAP-2 antibody (sec. Ab FITC) (magnification x400).

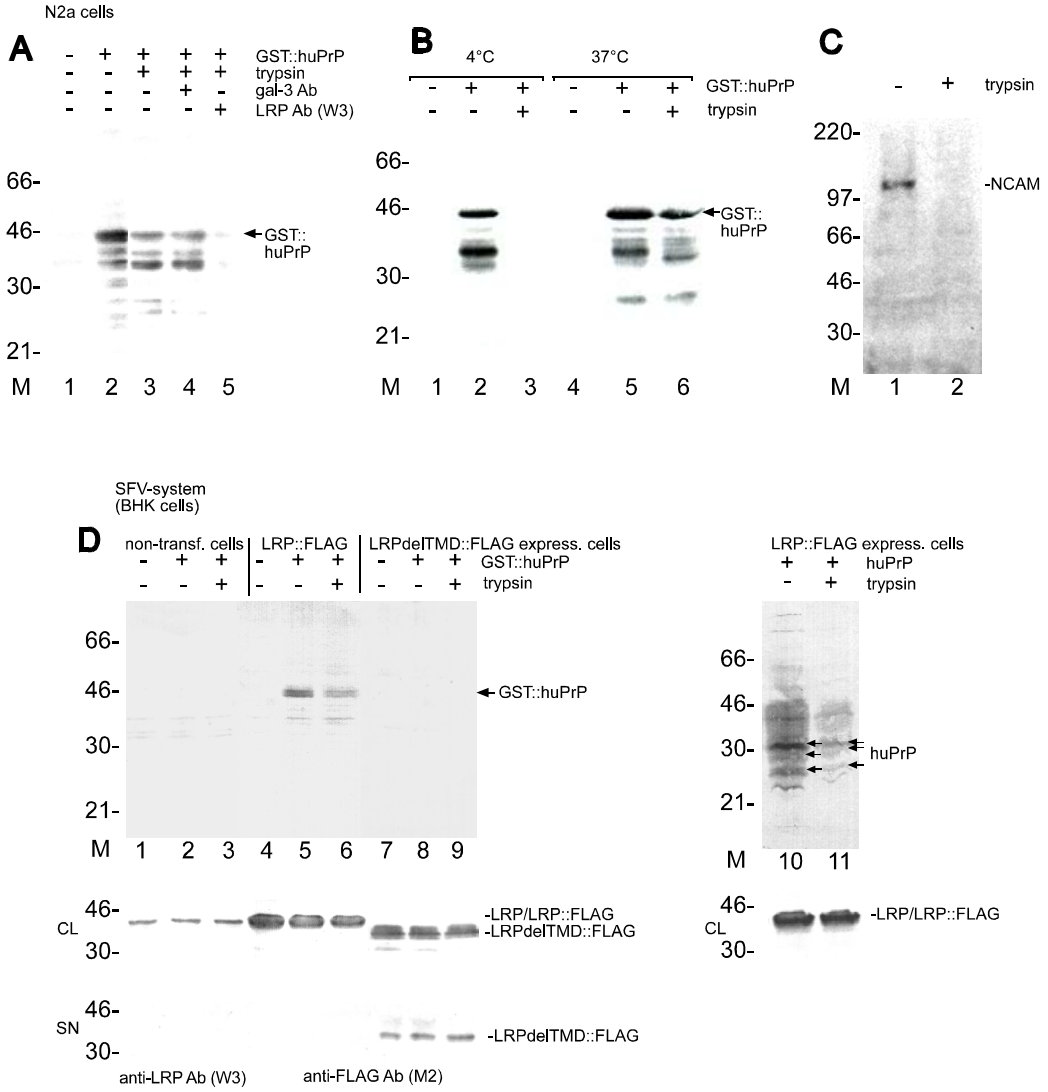

Fig. 5. LRP/LR-dependent binding and internalization of GST::huPrP by N2a cells. An LRP mutant lacking the transmembrane domain (LRPdelTMD) totally abolishes PrP binding and internalization on BHK cells. (**A**) Internalization of GST::huPrP by N2a cells. N2a cells not pre-incubated with antibodies (lane 1-3), pre-incubated with pAb gal-3 (dilution 1:5; lane 4), pAb LRP W3 (dilution 1:5; lane 5) were incubated with 8 µg/ml of GST::huPrP (lanes 2-5). Non-treated cells (lanes 1 and 2) and trypsin-treated cells (lanes 3-5) were analysed by western blotting employing mAb 3F4. (**B**) Temperature-dependent internalization of PrP by N2a cells. Cells were incubated with 8 µg/ml of GST::huPrP (lanes 2, 3, 5 and 6) at 4°C (lanes 1-3) and 37°C (lanes 4-6). Non-treated cells (lanes 1, 2, 4 and 5) and trypsin-treated

cells (lanes 3 and 6) were analysed by western blotting employing mAb 3F4. (**C**) Total cell extracts from trypsin-treated N2a cells (lane 2) or non-treated cells (lane 1) were analysed by western blotting employing pAb N-CAM directed against the neuron-specific cell adhesion molecule (N-CAM). (**D**) Binding and internalization of GST::huPrP by BHK cells hyperexpressing full-length LRP::FLAG or an LRP mutant lacking the transmembrane domain (aa 86 to 101) termed LRPdelTMD::FLAG. BHK cells either non-transfected (lanes 1-3), hyperexpressing LRP::FLAG (lanes 4-6, 10 and 11) or LRPdelTMD::FLAG (lanes 7-9) by the SFV system were incubated with either 5 µg/ml of GST::huPrP (lanes 1-9) or 5 µg/ml huPrP (generated in the SFV-system, lanes 10 and 11). Total cell extracts from non trypsin-treated (lanes 1, 2, 4, 5, 7, 8, and 10) and trypsin-treated cells (lanes 3, 6, 9 and 11) were analysed by western blotting employing the mAb 3F4 (upper panels), pAb LRP W3 (middle/lower panels, lanes 1-3, 10 and 11) or the mAb FLAG M2 (middle/lower panel, lanes 4-9). CL, crude lysate; SN, supernatant.

Supplementary Material section (SMS) :

published online as:
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The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO Journal*, **20**, 5863-5875

Materials and methods

Recombinant pSFV plasmid constructions and SFV-mRNA generation

The LRP (aa1-aa295) encoding cDNA was PCR-amplified from pCEP4 introducing *Bam*HI (5') and *Xma*I (3‘) restriction sites at the 5' and 3' ends. The 943 bp fragment encompassing Kozak sequence/AUG (5') and a FLAG-tag (3') was cloned into the pSFV1 (Liljestrom and Garoff, 1991) resulting in *pSFV1-LRP::FLAG*. *pSFV1-LRPdelTMD::FLAG* was generated by the QuikChange^{TM} site-directed mutagenesis method (Stratagene) employing pSFV1-LRP::FLAG DNA as template. The human prion ORF (Krasemann et al., 1996) was subcloned into pBK-CMV (Stratagene, LA Jolla, USA) and subsequently pBS+ (Stratagene, La Jolla, USA) to create compatible *Bam*HI sites. These *Bam*HI fragments were subcloned into pSFV1 resulting *in pSFV1-huPrP1-253*. All constructs were confirmed by dideoxy sequencing. DNAs pSFV3-lacZ (Life Technologies), pSFV1-huPrP1-253, pSFV1-LRP::FLAG and pSFV1-LRPdelTMD::FLAG were linearized with *Spe*I following purification by phenol-chloroform extraction. 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 the transcripts was verified by agarose gel electrophoresis. RNA was stored at -20°C.

BHK 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 FCS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5 % CO_2. Transfection and co-transfection (1:1) were carried out with individual SFV-RNAs by electroporation using a BioRad Gene Pulser. 1/10 volume of the electroporated cells ($8x10^5$) was diluted in complete growth medium and plated on 35 mm cell culture dishes for cell binding assays or wells containing a sterile glass coverslip for immunofluorescence microscopy. Cells were incubated for 24 h. Transfection efficiencies as determined by transfecting SFV3-lacZ control RNA followed by X-gal staining were 90-100% for BHK cells.

Construction of pCR3-uni™-huPrP1-253 and transfection of Hela cells

PCR primers PrPint2an and PrP813ac were used to amplify a 826 bp fragment encompassing the entire human PrP open reading frame from genomic human DNA. Subcloning of this fragment into pCR3-uni™ (Invitrogen) encompassing the CMV promoter resulted in pCR3-uni™-huPrP1-253, confirmed by didesoxy sequencing (Jaegly et al., 1998). The human epitheloid carcinoma of cervix Hela (ATCC: CCL2) cells were cultured in RPMI 1640 supplemented with 10% heat-FCS, 100 µg/ml of penicillin/streptomycine and 1% L-glutamine. Confluent cells were transiently transfected overnight in OPTIMEM™ (BRL) medium on 8 well Labtek® Chambers using 2 µg of DNA in Lipofectin® Reagent mixture (Life/technologies). Cells were harvested 3 days after transfection in growing medium and then fixed in 4% PFA. Transfection efficiencies were 10-20%.

Tissue culture of N2a, N2a [MHM2], MNB, NT2 and Sf9 cells

N2a, N2a [MHM2] and MNB mouse neuroblastoma cells, human NT2 cells were maintained in DMEM medium containing 10 % FCS, 1 % glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycine. *Spodoptera frugiperda* (Sf9) insect cells were maintained in Sf900II serum-free medium.

Primary mouse cortical cultures

Primary mouse cortical cells were established from 15 day old mouse embryos. Cortices were dissected in PBS Ca^{2+}/Mg^{2+} free supplemented by 5% glucose, carefully freed of meninges and incubated in trypsin/EDTA solution for 10 min at 37 °C. The trypsin was inactivated by incubation in DMEM containing 4.5 g/l glucose, Glutamax-I and 1% FCS. Cells were then dissociated mechanically. The suspension was pelleted by centrifugation and resuspended in DMEM containing B27 and 3% FCS. Eight-well Labtetk® culture slides coated with 10 µg/ml of poly D lysine were seeded at $3x10^5$ cells per well in 0.3 ml of DMEM B27 supplemented by 3% FCS and 100 µg/ml penicillin, 100 µg/ml streptomycin. Cultures were kept in a water saturated incubator with an atmosphere of 95% air 5% CO2 for 2 days. Medium was then changed for serum free containing DMEM supplemented with B27 components. After 2 days, cells, which were immunocytochemically defined as 95 % pure in neurons (according to MAP2 immunolabelling) and containing less than 5% glial cells were exposed to recombinant PrP. PrP knock-out mice used to establish $PrP^{0/0}$ neuronal cultures were kindly provided by C. Weissmann (University of Zürich, Switzerland).

Construction of baculovirus expression vectors, protein synthesis in the Baculovirus, E.coli and SFV system

cDNA encoding huPrP23-230 (H. A. Kretzschmar, Munich) was generated by PCR and cloned into the transfer vector pAcSecG2T via *Bam*HI (5´) and *Eco*RI (3´) resulting in *pAcSG2T-huPrP23-230*. Recombinant viruses were generated by co-transfection of the transfer vectors with linearized viral DNA (Baculogold; Pharmingen). cDNA encoding for haPrP-peptide 23-89 was cloned via *Bam*HI (5') and *Eco*RI (3') into pAcSG2T resulting in pAcSG2T::PrP23-89. Recombinant 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). All recombinant proteins were dialyzed against 20 mM Hepes, pH 7.4. Authentic PrP^c was prepared from hamster brain membrane fractions as previously described (Meyer et al., 1986). Highly glycosylated wild-type human PrP was expressed in BHK cells transfected with pSFV1-huPrP1-253 RNA. 48 h post transfection the cells were harvested, washed once with PBS and then lysed in PBS supplemented with 0.1% TritonX-100 by repeated freezing and thawing. The crude lysate was obtained by centrifugation at 14 000 rpm

4°C for 15 min and dialyzed against 20 mM Hepes, pH 7.4. Purified Galectin-3 produced in *E.coli* was a generous gift from Prof. Hans-Joachim Gabius, Munich.

Far-UV Circular dichroism analysis

GST::huPrP23-230 was dialyzed against 20 mM Tris.HCl pH 9.0, 5 mM dithiothreitol, 1 mM EDTA followed by dialysis against 10 mM sodium phosphate buffer, pH 7.4. CD spectra were recorded on a Jasco model J-710 spectropolarimeter. Measurements were carried out in a 1 mm path-length cylindrical cuvette at room temperature at 190 -260 nm. Typically 10 spectra were recorded at a scan speed of 20 nm/min with a step resolution of 0.1 nm.

PrP-Binding Assays followed by immunofluorescence analysis and confocal microscopy

For competition studies the cells were either pre-incubated for two hours with the individual antibody diluted to various concentrations in culture medium or incubated with recombinant protein which was pre-incubated with the antibody (inoculum saturation). In case of pre-incubation medium was replaced and cells were incubated overnight with the indicated amounts of GST::huPrP. Cells were then washed three times with PBS and prepared for IF. Cells were seeded half confluently on coverslips, grown overnight for attachment, washed three times with PBS and fixed with 4% paraformaldehyde. Non-permeabilized cells were fixed with 2 % paraformaldehyde. After rinsing 3 times with PBS, cells were permeabilized for cytoplasmic staining with 0.2 % Triton X-100 (10 min/4 °C). The preparation was saturated with a 10 % FCS solution (in PBS) for 1 h at room temperature, washed and incubated with the primary antibodies diluted in PBS with 10 % FCS for 1 h at room temperature. Staining of the individual protein was performed with the indicated mono- or polyclonal antibodies. After washing 3 times with PBS the preparations were diluted in saturation buffer and incubated in the dark for 45-60 min with the individual secondary antibodies (goat anti mouse or goat anti rabbit) conjugated with FITC, Texas Red, Cy2 (green) and Cy3 (red). For nuclear staining 1 µg/ml DAPI for 10 min at RT was used. Please note that both the primary and secondary antibodies were added after fixing the cells. The coverslip was mounted with aqueous mounting medium (Fluoromount®) and the slides were examined using a axioviert fluorescence microscope (Zeiss) with appropiate filters or a Zeiss confocal microscope. Immunofluorescence images were processed using Metamorph software®.

FACS analysis (flow cytometry)

Single-cell suspensions were prepared in PBS, 2% fetal calf serum, 20 mM EDTA, 0.01% sodium azide (FACS buffer). For flow cytometry, cells were incubated with the first antibody at concentrations of ~1 µg/10^6 cells for 15 minutes at room temperature. Cells were washed in FACS buffer before incubation with FITC-conjugated anti-rabbit IgG for 15 minutes at room temperature. After washing in FACS buffer, data acquisition and analysis were performed with an EPICS XL-MCL (Coulter) flow cytometer. Dead cells were gated out by forward and side scatter properties. Polyclonal anti-gal-3 and polyclonal anti-LRP antibody (W3) were used as primary antibodies, rabbit IgG (Sigma) was used for isotype controls.

PrP-Binding assay in cell culture followed by Western Blotting

$3x10^5$ N2a cells and $8x10^5$ BHK cells (either non-transfected or transfected with recombinant SFV RNAs as described above) were each seeded on 6-well plates and incubated at 37 °C. For competition studies cells were pre-incubated for two hours with the indicated antibody. Medium was replaced after pre-incubation. Cells were then incubated in medium containing different amounts of the individual protein for 18 h at 37°C or 4°C (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 CaCl2, 3 mM MgCl2, 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 indicated primary antibody and peroxidase-coupled secondary antibodies.
In order to analyze secreted proteins from the medium, the supernatants of transfected or non-transfected BHK cells were centrifuged at 1100 rpm for 10 min and the supernatants were analyzed by Western blotting.

Isolation of plasma membranes

Plasma membrane preparations from 10^8 N2a and BHK cells, the latter either non-transfected or transfected with SFV-LRP-FLAG RNA, were prepared as described (Vleurick et al., 1999).

Calculation of binding curves for recombinant PrP to cells and determination of the k_D for the interaction of PrP with LRP/LR

Western Blots have been optically scanned employing a flat bed scanner. The optical density of the individual bands has been measured employing the NIH-Image software program. The resulting values represent square pixels. Background values have been subtracted from the values measured. Standard curves have been determined to prove that values range within the linear area. The dissociation constant (K_D) for the interaction of PrP with LRP/LR on the cell surface has been calculated from the binding curve in Figure 3F by using the Prism 3 software program. The data were analyzed by non-linear regression using a hyperbolic curve fitting option.

Trypsin cell treatment for PrP internalization studies

Cells were incubated with recombinant proteins as described above. After 18 h at 37°C, cells were washed several times with PBS and incubated with trypsin (250 µg/ml) at 37°C for 10 min. The reaction was terminated by addition of the cell specific growth medium. Cells were collected by centrifugation at 1100 rpm for 10 min, washed twice with PBS, lysed and analyzed by Western blotting.

Antibody saturation

In order to prove specificity of the polyclonal anti-LRP-Ab (W3), W3 was rotated over night with rec. GST::LRP immobilized to glutathione sepharose beads at 4 °C. The supernatant failed to recognize LRP by Western Blotting and was employed in IF-analyses on N2a{MHM2) and transfected BHK cells.

Antibodies

Polyclonal Ab anti-LRP W3 (Rieger et al., 1997), mAb directed against aa167-243 and aa285-295 of LRP, respectively, (J.P. Houchins, Minneapolis), the mAB 3B5 (G. Hunsmann), pAb GST (Santa Cruz Biotech. Inc.) and mAb GST (Sigma), mAb 3F4 (Senetek, USA), mAb SAF70 against aa 140 to 180 of PrP (Service de Pharmacologie et Immunologie, CEA, Saclay, France), pAb JB007 (Service de Neurovirologie, CEA, France), pAb against PrP (M-20) (Santa Cruz Biotech. Inc.), pAb anti-laminin (Roche Diagnostics), mAb anti-lutheran protein (J.-P. Cartron, INTS, France), pAb anti-GFAP (Roche Diagnostics), pAb MAP-2

(Santa Cruz Biotech. Inc.), mAb anti-VLA-6 CD49-f (Immunotech), Ab anti-N-CAM (Santa Cruz Biotech. Inc.), Abs anti-Hsp60/70/90 (Sigma, Munich), mAb anti-FLAG M2 (Sigma), pAb anti-FLAG (Santa Cruz Biotech. Inc.), pAb anti-galectin-3 (H.-J. Gabius, Munich), secondary FITC, Cy2 (carbocyanine), Cy3 (indocarbocyanine) and Texas Red conjugated antibodies (used at 1:100 dilutions) (Jackson Laboratories and Southern Biotechnology, respectively) were used.

CHAPTER IV

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.

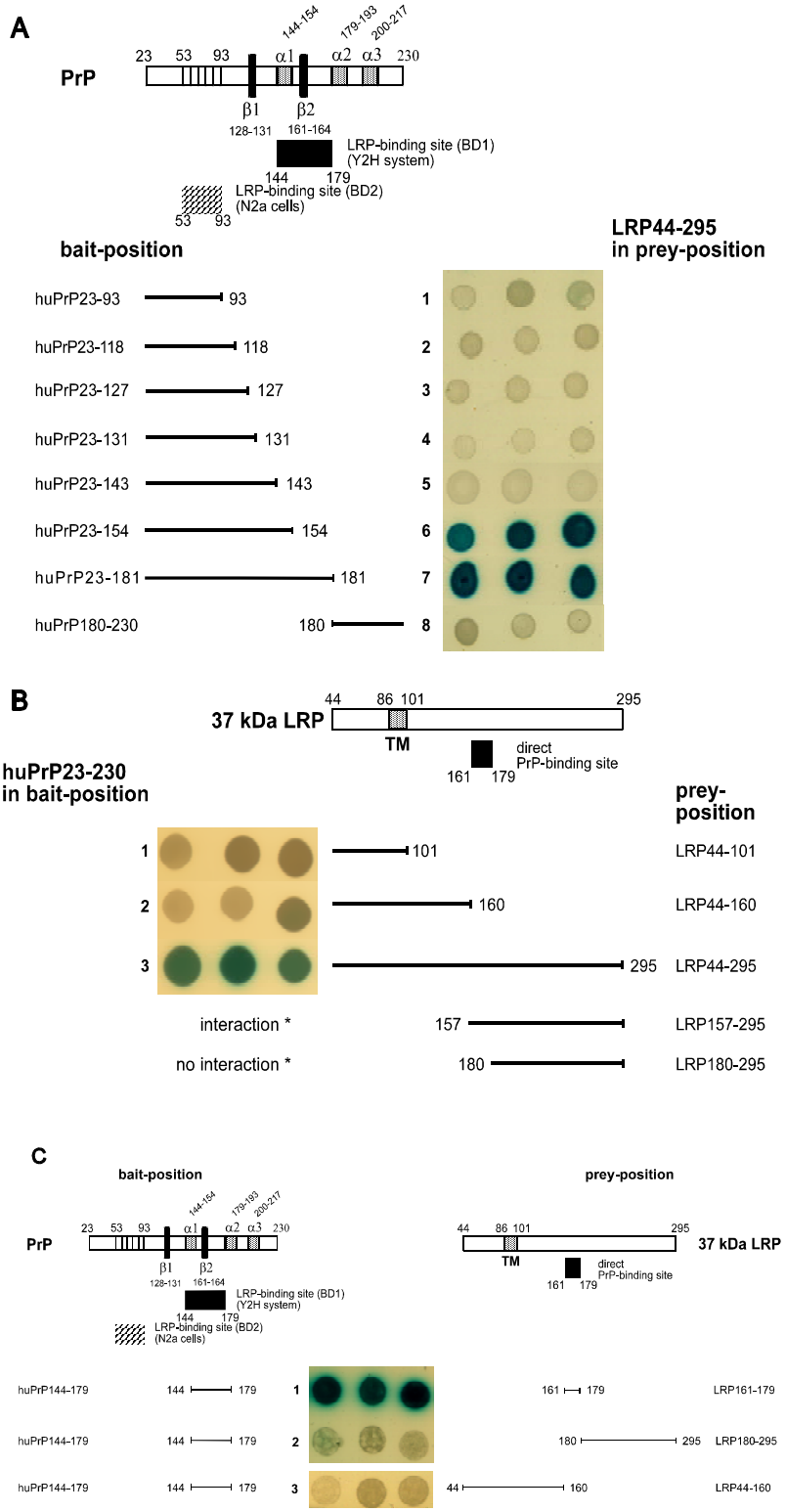


Fig. 1. Identification of direct PrP-LRP interaction domains. (**A**) Identification of the direct PrP-LRP interaction domain on PrP. HuPrP23-93 (row 1), huPrP23-118 (row 2), huPrP23-

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.

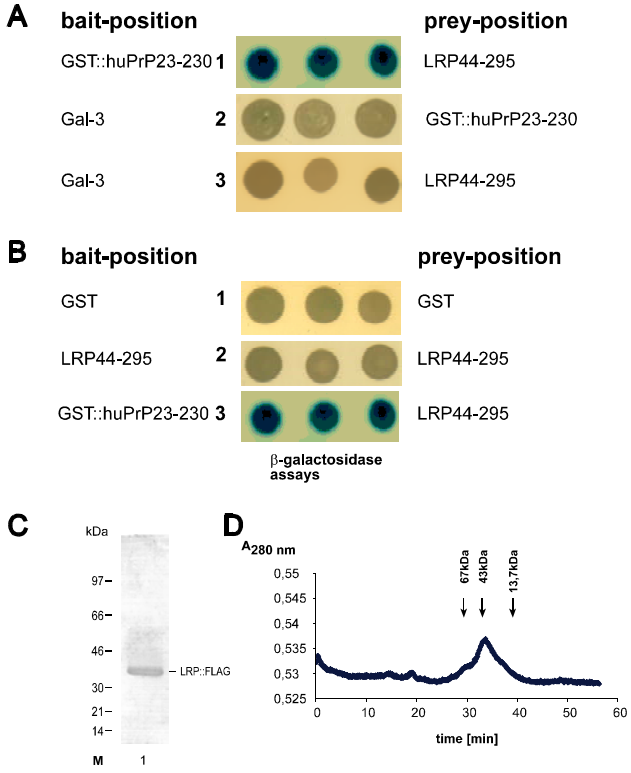


Fig.2. LRP fails to interact with itself in the yeast two-hybrid system and appears monomeric by native size-exclusion chromatography. Gal-3 fails to interact with PrP and LRP. (**A**) huPrP23-230 and LRP fail to interact with the β-galactoside lectin gal-3 in the yeast two-hybrid system. huPrP23-230 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.

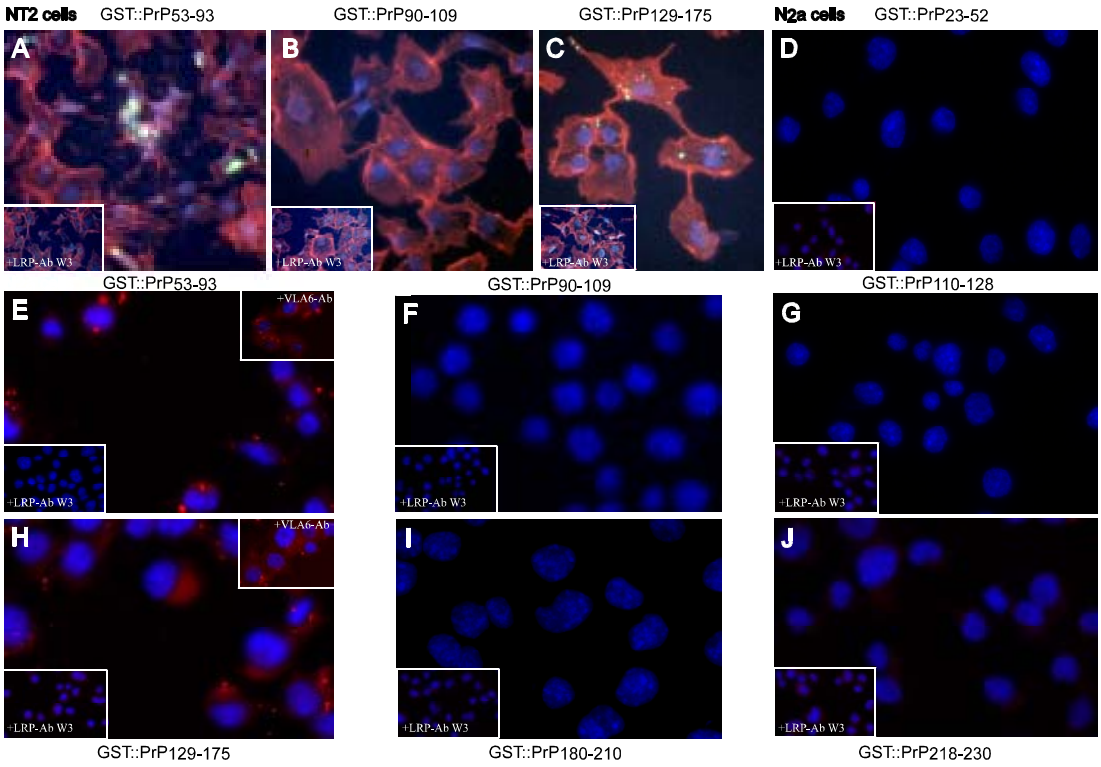
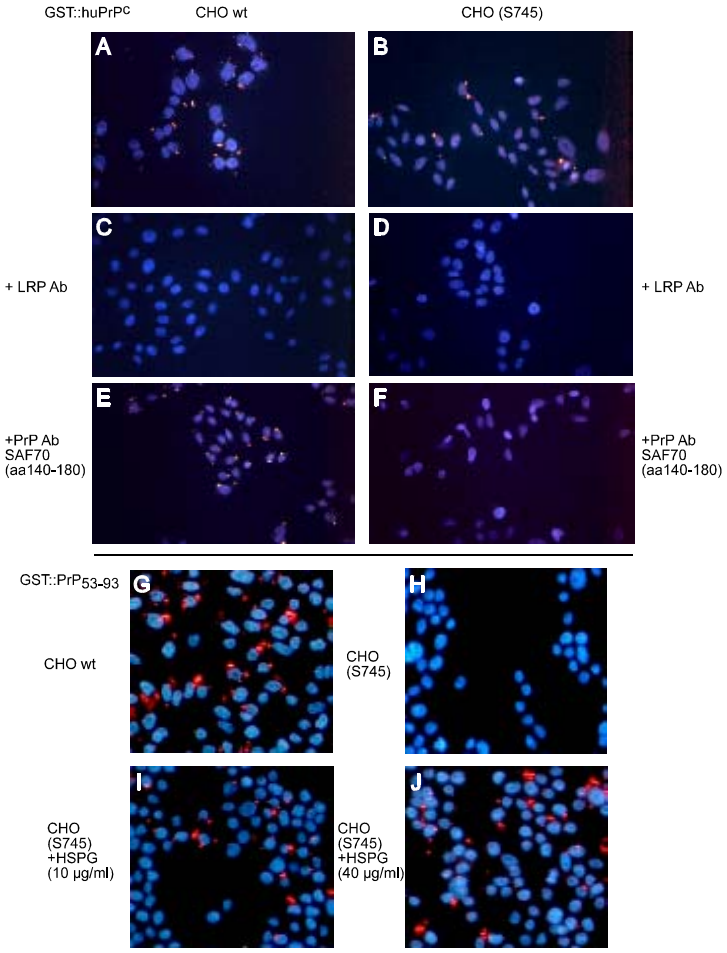
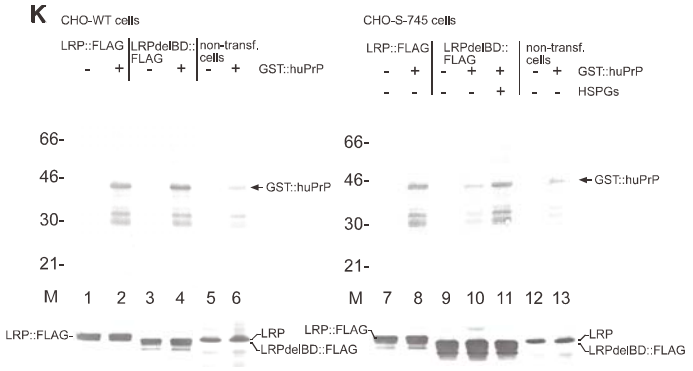


Fig. 3. Identification of PrP-interaction domains for LRP/LR by binding assays with rec. prion peptides on NT2 and N2a cells. (A-C) Binding to NT2 cells. NT2 cells were incubated with PrP peptides fused to GST in the absence (A-C) and presence (insets in A-C) of the preincubated pAb LRP W3 (dilution 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).


GST::huPrPc
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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CHO-S-745 cells
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LRPdelBD::FLAG
non-transf. cells
GST::huPrP
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LRP
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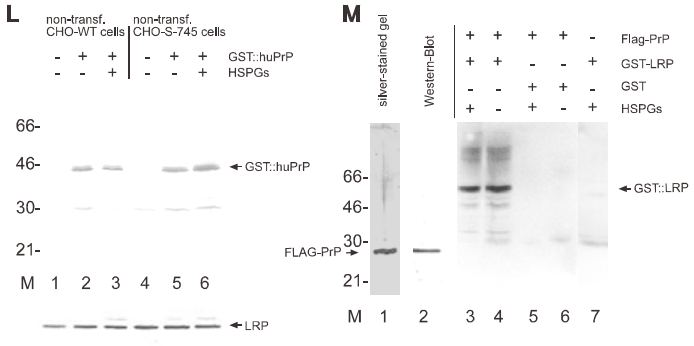
L
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66-
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LRP
M
silver-stained gel
Western-Blot
Flag-PrP
GST-LRP
GST
HSPGs
GST::LRP
FLAG-PrP
M 1 2 3 4 5 6 7

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).

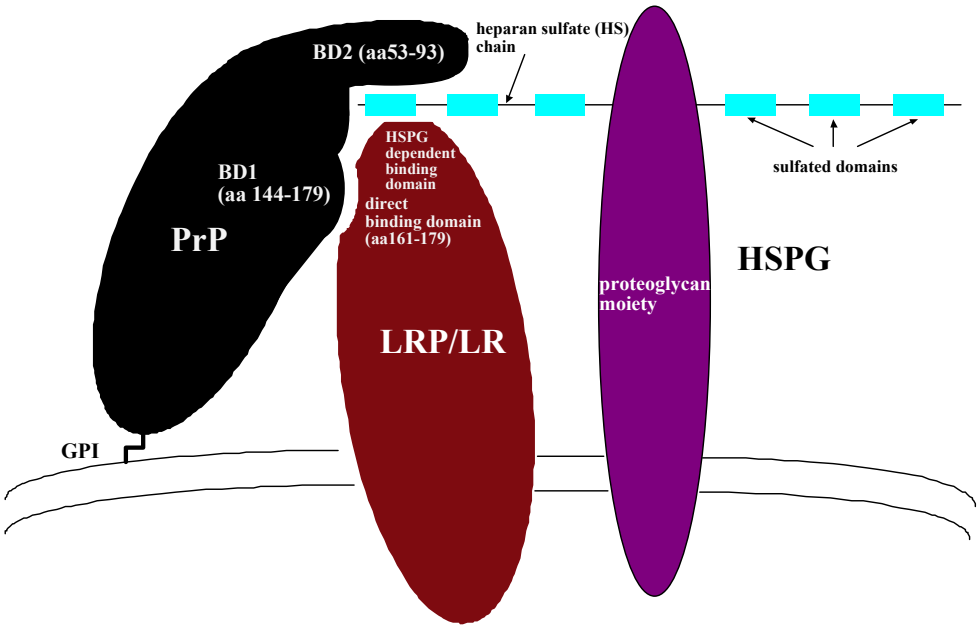

Fig. 5. Model for the function of LRP-LR as the receptor for PrP. The PrP molecule binds to LRP-LR via PrP^{LRPbd1} and PrP^{LRPbd2}. PrP^{LRPbd2} (aa 53-93) is dependent on the presence of a heparan sulfate arm of a HSPG molecule whereas PrP^{LRPbd1} (aa 144-179) interacts directly with LRP-LR (as shown 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 V

Recombinant human prion protein mutants huPrP D178N/M129 (FFI) and huPrP+9OR (fCJD) reveal proteinase K resistance

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The recombinant human prion protein mutants huPrP D178N/M129 (FFI) and huPrP+9OR (fCJD) reveal proteinase K resistance.

Abstract

The Semliki Forest virus (SFV) sytem was used to overexpress human wild-type and mutant prion proteins as well as FLAG-tagged human and bovine PrP in mammalian cells. The application of recombinant SFV vectors allowed a high-level production of highly glycosylated prion proteins with a molecular weight ranging from 25 to 30 kDa for recombinant wild-type human PrP and from 26 to 32 kDa for wild-type bovine PrP. Further, we report here about the generation of recombinant mutant prion proteins which are associated with inherited human prion diseases such as fatal familial insomnia (FFI) and Creutzfeldt-Jakob disease (CJD). Both mutated variants, the FFI-associated PrP carrying a mutation at amino acid (aa) position 178 and the CJD-linked form containing an insertion of nine additional octarepeats reveal proteinase K (PK) resistance, one of the most typical biochemical properties characteristic for the infectious scrapie isoform of the prion protein. In contrast, recombinant wild-type PrP was completely protease K sensitive when expressed in SFV-transfected BHK cells. The subcellular location of both PrP mutants at the cell surface and in intracellular compartments of transfected BHK cells was similar to that of wild-type PrP without any significant differences regarding the cellular distribution and expression level. In order to purify recombinant human and bovine PrP from cell lysates, a FLAG-tag was introduced either at the N-terminus behind the signal peptide or at the C-terminus close to the adhesion site of the GPI anchor. N-terminal insertion did not influence trafficking of the FLAG-tagged protein to the cell surface whereas insertion close to the GPI attachment site clearly affected the transport of PrP to the cell membrane probably resulting in the retention within the secretory pathway. All FLAG-tagged prion proteins were expressed high efficiently in BHK cells showing the typical glycosylation pattern allowing the rapid and simple purification via anti-FLAG antibody chromatography.

Introduction

The causative agent of transmissible spongiform encephalopathies (TSEs) is termed prion defined as a proteinaceous infectious particle (Prusiner, 1982). Prion diseases (for review see (Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann and Aguzzi, 1997)) belong to a new class of fatal neurodegenerative disorders affecting humans and animals. The key event in the development of disease are conformational changes of the cellular prion protein, PrP^{c}. The misfolded, disease-associated form is termed PrP^{Sc}, a partially proteinase K resistant isoform with a high β-sheet content compared to the proteinase K sensitive and predominantly α-

helical PrP^c (Caughey *et al*., 1991). TSEs may occur spontaneously, inherited or transmitted. Human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS) (for review see (Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann and Aguzzi, 1997). Whereas 85% of all CJD cases occur sporadically in the absence of any mutations, FFI, GSS and the remaining 15% of CJD are dominantly inherited diseases caused by defined mutations within the *Prn*-p gene on chromosome 20 (Masters, 1981; Sparkes *et al*., 1986). Genetic linkage or association has been identified for several amino acid substitution in the C-terminus of the PrP as well as for octarepeat insertions within the N-terminal part of the molecule. Here, the spontaneous conversion of PrP^c to PrP^{Sc} in the absence of exogenous prions has been supposed (Young, 1999) but the exact mechanism by which these mutations provoke the conformational changes is still unknown. Destabilization of the PrP^c structure or effects on the thermodynamic stability of PrP are suggested (Liemann and Glockshuber, 1999; Riek *et al*., 1998; Swietnicki *et al*., 1998; Zhang *et al*., 2000).

The physiological function of PrP still remains unclear. It has been proposed that PrP might play a role in synaptic processes (Collinge *et al*., 1994), in the regulation of circadian activity rhythms and sleep (Tobler *et al*., 1996) and in copper transport (Hornshaw *et al*., 1995). Further on, a function in the survival of purkinje cells (Sakaguchi *et al*., 1996) was suggested but very recently, it has been reported that the upregulation of the prion-like protein termed doppel causes Purkinje cell degeneration in $Prnp^{0/0}$ mice instead of PrP depletion (Moore *et al*., 1999). More recently, a superoxide dismutase activity (Brown *et al*., 1999) and a role in signal transduction (Mouillet-Richard *et al*., 2000) of PrP has been suggested. Due to the absence of any phenotype for PrP described in various reports (Bueler *et al*., 1992; Lledo *et al*., 1996; Manson *et al*., 1994), the only confirmed role of PrP^c seems to be its necessity for the development of TSEs (Bueler *et al*., 1993). Cellular PrP is synthesized in the rough endoplasmic reticulum (rER) and is transported via the Golgi and secretory granules to the surface of neuronal cells where it is anchored to the plasma membrane by its glycosyl phosphatidylinositol (GPI) moiety (Rogers *et al*., 1991). Three different PrP glycoforms differing in their glycosylation degree have been observed on the cell surface: diglycosylated (70%), monoglycosylated (25%) and unglycosylated PrP (5%) (Caughey *et al*., 1989; Monari *et al*., 1994; Petersen *et al*., 1996). Recently, we identified the 37 kDa Laminin Receptor Precursor (LRP) as an interactor for the prion protein (Rieger *et al*., 1997) (for reviews see (Gauczynski *et al*., 2001a; Rieger *et al*., 1999)). Cell-binding and internalization studies on neuronal and non-neuronal cells have demonstrated that the 37-kDa/67-kDa laminin receptor

acts as the cell-surface receptor for the cellular prion protein (Gauczynski *et al*., 2001b). Direct and heparan sulfate proteoglycan (HSPG)-dependent interaction sites mediating the binding of cellular PrP to its receptor have been identified (Hundt *et al*., 2001). Cell culture experiments demonstrated the 37-kDa/67-kDa LR dependent binding and internalization of recombinant GST::human PrP generated in insect cells and glycosylated human PrP synthesized in BHK cells transfected with recombinant SFV-RNA (Gauczynski *et al*., 2001b). High-level expression and purification of recombinant, glycosylated prion proteins in mammalian cells is essential for a better understanding of the physiological function of PrP^{c} and biochemical processes responsible for familial prion diseases. The synthesis and study of wild-type as well as mutant PrP in cell culture systems allows a better insight into the biology of these proteins due to the presence of important organelles, membranes and other cellular cofactors which are necessary for the correct processing, trafficking and localization of the protein. Therefore, we used the Semliki Forest virus (SFV) system to express high amounts of glycosylated wild-type and mutant disease-associated prion protein in cultured mammalian cells. The SFV system supplies a multitude of advantages for the expression of recombinant proteins in mammalian cells: (i) a large-scale production for up to 72 hours post-transfection, (ii) a broad host range, (iii) modifications such as glycosylation in a correct and sufficient way and (iv) the easy and fast transfection procedure with *in vitro*-transcribed RNA. The Semliki Forest virus is an insect-borne alphavirus and belongs to the family of Togaviridae (Schlesinger, 1986). Its viral genome consists of capped and polyadenylated single-stranded RNA of positive polarity and encodes its own RNA polymerase. SFV expression vectors are based on a cDNA copy of the viral genome. Here, viral structural genes are deleted and replaced by the gene of interest. Due to the remaining viral replicase which leads to an efficient production of recombinant RNA within the cell, a high-level synthesis of the foreign protein proceeds (Liljestrom and Garoff, 1991).

In the present work, we have generated recombinant disease-related mutant isoforms of human PrP in BHK cells transfected with rec. SFV RNAs. The FFI-associated mutant PrP D178N/M129 and the CJD-related PrP+9OR were efficiently and highly glycosylated expressed in cultured cells. We further examined biochemical features such as glycosylation status and proteinase K resistance of recombinant mutated PrP in comparison to recombinant, wild-type PrP as well as their subcellular localization in transfected BHK cells. We observed that both mutants were PK resistant at 8 µg/ml PK sharing this biochemical hallmark with infectious PrP^{Sc}. Therefore, the SFV system does not only result in expression system for

high-level production of glycosylated prion proteins, but may also function as a cell culture model for inherited human prion diseases.

To facilitate purification and to introduce an additional epitope for immunodetection, we introduced a FLAG-tag at different positions of the cellular human and bovine PrP. FLAG-tag insertions at the C-terminus of PrP located two amino acids N-terminally of the GPI-anchorage site, allowed the purification of predominantly diglycosylated human and bovine PrP from cell lysates by anti-FLAG antibody affinity chromatography.

Results

Expression of glycosylated wild-type and mutant prion proteins in BHK cells transfected with recombinant Semliki Forest virus (SFV) RNAs

In order to produce high amounts of recombinant prion proteins in mammalian cells, we used the Semliki Forest virus (SFV) expression system which is based on a cDNA copy of the viral replicon. Transfection efficiency of Baby hamster kidney (BHK) cells used throughout all our experiments was almost 100% (data not shown) warranting the high-level production of glycosylated prion proteins. BHK cells were transiently transfected with recombinant SFV RNAs encoding full-length human PrP encompassing aa 1 to 253, human PrP containing the FFI-associated mutation at codon 178 (D178N,129M), human PrP with the CJD-linked insertion of nine octarepeats and wild-type bovine PrP from aa 1 to 264. Western blot analysis of crude lysates demonstrates the expression of di-, mono- and low amounts of non-glycosylated forms of PrP with a molecular weight ranging from 25 to 30 kDa for wild-type and FFI-associated human PrP, from 31 to 37 kDa for huPrP+9OR and from 26 to 32 for bovine PrP (Figure 1, lane 1, 3, 5 and 7), respectively. Also high-molecular, i.e. hyper-glycosylated forms of cellular and mutant PrP were synthesized and detected by the mAb anti-PrP 3B5 (Figure 1). To confirm the glycosylation state of recombinant prion proteins, cell lysates were treated with N-glycosidase F leading to a decrease in di- and monoglycosylated forms accompanied by an augmentation of the non-glycosylated PrP (Figure 1, lane 2, 4, 6 and 8).

Proteinase K status of recombinant cellular PrP and disease-associated PrP variants

The partial resistance to proteinase K is a major hallmark of PrP^{Sc}. In contrast, cellular PrP is completely pK sensitive. To investigate whether recombinant FFI- and CJD-associated PrP isoforms synthesized in SFV-transfected BHK cells share PrP^{Sc}-like properties, proteinase K

was added to total cell extracts at increasing concentrations of 2, 4 and 8 µg/ml followed by incubation at 37 °C for 30 minutes (Figure 2). Both mutant PrPs, the FFI-associated PrP as well as the CJD-related insertion mutant, were resistant towards PK amounts up to 8 µg/ml, yielding PK-resistant fragments with molecular weights ranging from 23 to 25 kDa (Figure 2, lanes 8 and 12). In contrast, wild-type PrP was completely digested after treatment with 8µg/ml PK (Figure 2, lane 4).

Cellular localization of recombinant prion proteins in transfected BHK cells

To proof, whether recombinant prion proteins, either wild-type or mutant, are transported to the surface of transfected BHK cells, non-permeabilized cells were analysed by immunofluorescence (IF) microscopy. In cells expressing wild-type human or bovine PrP, the protein traffics to the cell surface where it appears partially in a punctuated manner (Figure 3B and E). Virtually all cells synthesizing PrP+9OR (Figure 3C) or PrP-FFI (Figure 3D) showed similar staining patterns and comparable expression levels on the cell surface suggesting that mutated PrP variants are correctly processed to the cell surface. To visualize PrP in the interior of transfected cells, cells were permeabilized prior to immunostaining. Expression of wild-type human and bovine PrP (Figure 3B and E, insets) as well of both mutated PrPs (Figure 3D and E, insets) revealed a wide-spread staining pattern including areas surrounding the nucleus and regions of the cytoplasm. Thus, regarding the subcellular localization, no differences between PrP mutants and the cellular forms were detectable. Cells transfected with SFV RNA encoding the viral replicase only showed no detectable PrP staining neither on the cell surface nor in the interior of permeabilized cells (Figure 3A and inset) suggesting that BHK cells express no or only marginally amounts of endogenous PrP^{C}.

Expression of glycosylated FLAG-tagged prion protein

To facilitate purification of human and bovine prion proteins and to introduce an additional epitope for immunodetection, we inserted a FLAG-tag at the C-terminus near the GPI-anchor adhesion site resulting in the constructs huPrP227FLAG and boPrP239FLAG and at the N-terminus behind the signal peptide resulting in huPrP22FLAG (Figure 4A). FLAG-tagged PrP isoforms were expressed highly glycosylated in transfected BHK cells (Figure 4B, lanes 1-3 and 4C, lanes 1 and 2). To confirm the glycosylation state, samples were deglycosylated by treatment with N-glycosidase F resulting in augmentation of the non-glycosylated PrP forms (Figure 4B, lanes 4-6 and 4C, lanes 3 and 4).

Cellular localization of FLAG-tagged prion proteins in transfected BHK cells

To proof, whether the FLAG tagged prion proteins are transported to the surface of transfected BHK cells, non-permeabilized cells were stained with anti-PrP antibody 3B5 and analysed by IF microscopy. As already shown in Figure 3, human and bovine wild-type PrP were efficiently transported to the cell surface of transfected BHK cells (Figure 3B and E; Figure 5B and E). Due to the overexpression of recombinant protein in rec. SFV RNA-transfected cells, wild-type human and bovine PrP were also detectable inside permeabilized cells (Figure 5B and E, insets). Permeabilization of transfected cells prior to immunostaining reveals intracellular deposits of both, wild-type and FLAG-tagged, prion proteins (Figure 5, insets). Whereas huPrP22FLAG was detectable on the cell surface at comparable levels (Figure 5C), both prion proteins carrying the FLAG-tag insertion close to the GPI-attachment site were only weakly transported to the surface of transfected BHK cells (Figure 5D and F) suggesting that the FLAG-tag at this position hampers trafficking of PrP to the cell surface. Indeed, most of the protein remains within the interior of the cell as shown by IF analysis on permeabilized cells (Figure 5D and F, insets). BHK cells transfected with SFV RNA encoding for the viral replicase only showed neither surface nor intracellular staining (Figure 3A and inset) confirming that no or only low amounts of cellular PrP are synthesized by BHK cells.

Purification of glycosylated FLAG-tagged PrP

Purification of FLAG-tagged prion proteins was performed by anti-FLAG antibody chromatography. FLAG-tagged proteins from cell lysates were immobilized to anti-FLAG M2-conjugated agarose beads (Figure 6A and B, lanes 2). Silver stained SDS gels revealed that mainly the diglycosylated PrP form was purified to homogeneity by this one-step purification process (Figure 6C). Both kinds of recombinant proteins were recognized by the PrP specific antibody 3B5 (Figure 6A and B, lanes 3).

Discussion

Synthesis of wild-type and mutant prion proteins in mammalian cells by the Semliki Forest virus system

In order to unseal the mystery about the physiological function of the cellular prion protein, great efforts have been done to get a better understanding of the biology and structure of PrP^{c}. Different expression systems have been applied to produce recombinant prion proteins

in prokaryotic, insect, yeast or mammalian cells. Expression of Syrian golden hamster prion protein in *E. coli* and in Baculovirus-infected insect cells using the glutathione S-transferase (GST) fusion system were described (Volkel *et al*., 1998; Weiss *et al*., 1996; Weiss *et al*., 1995). Although these systems resulted in large-scale protein production, only unglycosylated or marginal glycosylated PrP has been obtained. Thus, we decided to establish the SFV-system for high-level expression of different wild-type and mutant prion proteins in mammalian cells. As expected, rec. glycosylated PrP was synthesized in BHK cells transfected with recombinant SFV RNAs. The typical glycosylation pattern consisting of unglycosylated, mono- and diglycosylated PrP was observed when we investigated the total cell extracts by western blot analysis. The presence of N-linked oligosaccaride chains was confirmed by N-glycosidase F treatment which led to deglycosylation of PrP resulting in the generation of mainly the non-glycosylated PrP form.

Processing and trafficking of wild-type and mutated human prion proteins

Correctly processed PrP traffics to the cell surface where it is anchored to the plasma membrane by its GPI-moiety (Rogers *et al*., 1991). We investigated the subcellular location of recombinant wild-type and disease-associated mutant PrP by IF microscopy. Very recently, it has been observed that mutant PrP molecules which are linked to familial prion diseases partially retained in the endoplasmic reticulum (ER) (Ivanova *et al*., 2001). This led to the conclusion that several pathogenic mutations i.e. point and insertion mutations within the *Prn*-p gene exert influence on the trafficking of PrP and that protein quality control might play an important role in TSEs (Ivanova *et al*., 2001) (for summary see table 1). For several mutations an additional mode of membrane association resulting in the resistance against enzymatic cleavage of the GPI-anchor was reported (Lehmann and Harris, 1995; Lehmann and Harris, 1996a), a phenomen which is also typical for infectious PrP^{Sc}. Previous studies have already described that mutant prion proteins are inefficiently processed to the cell surface and accumulate in intracellular compartments such as ER, Golgi and endosomes/lysosomes (Capellari *et al.*, 2000; Jin *et al*., 2000; Petersen *et al*., 1996; Singh *et al*., 1997; Zanusso *et al*., 1999) (for summary see table 1). In contrast, IF analysis of non-permeabilized, transfected BHK cells expressing huPrP-FFI or huPrP+9OR revealed similar surface staining in comparison to cells expressing wild-type human PrP at their cell surface. In addition, the intracellular staining of wild-type as well as both mutant PrP isoforms revealed the same wide-spread pattern encompassing nearly the entire interior of the cell. This effect might be based on the SFV-system due to the hyperexpression of recombinant protein

while competing out the host translation machinery. Therefore, it might be difficult to detect subcellular trapped PrP in cells overexpressing this protein. However, we cannot exclude that mutant PrP molecules are partially retained in cellular compartments such as the ER.

Proteinase K resistance of mutated human prion proteins

One major biochemical characteristic of PrP^{Sc} represents its resistance to proteinase K (PK). Some mutant isoforms of the mouse prion protein carrying mutations homologous to familial human prion diseases were produced in stably transfected CHO cells and displayed scrapie-like biochemical properties such as proteinase K resistance (for summary see table 1) and detergent insolubility (Daude *et al*., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b). Furthermore, hamster insertion mutants encompassing additional octarepeats showed protease resistance when synthesized in cultured cells (table 1) (Priola and Chesebro, 1998). To investigate whether genetically-linked human PrP mutants expressed in transiently transfected BHK cells behave similar to PrP^{Sc}, total cell extracts were treated with PK at a final concentration of 2, 4 and 8 µg/ml for 30 min at 37°C. Under these conditions, we detected a protease-resistant fragment of FFI-associated and CJD-related human PrP with a molecular weight ranging from 23 to 25 kDa. Previous reports describing a partially PK resistance for disease-associated PrP used partly lower PK concentrations and/or shorter incubation times (for summary see table 1). The mouse PG14 PrP mutant encompassing nine additional octarepeats, was PK sensitive at a concentration of 2 µg/ml when synthesized in stably transfected BHK cells using the Sindbis replicon (Ivanova *et al*., 2001). In addition, the human PrP mutant D178N/M129 was totally degraded when digested with low amounts of PK for 1 hour or with 5 µg/ml PK for at least 5 minutes (table 1). It is difficult to compare the characteristics of mutant PrPs of different species, expression systems and cells used for generation and the diverse conditions of PK digestion. Employing the SFV system, we were able to synthesize glycosylated, cell-surface orientated and PK resistant (up to 8 µg/ml) human PrP mutants recommending its capability as a cellular model system for familial human prion diseases. Bioassays will prove whether these PK-resistant PrP molecules will harbor endogeneous infevtivity.

Processing, trafficking and purification of FLAG-tagged human and bovine prion proteins

In order to purify prion proteins rapid and simple from total cell extracts and to introduce an epitope suitable for specific immunogenic detection, a FLAG-tag was inserted within the

human PrP encoding sequence close to the GPI anchorage site at residue 227 or at the N-terminus following the signal peptide sequence at residue 22. A homologous FLAG-tag insertion within the bovine PrP sequence termed boPrP239FLAG was also constructed. The locations of the FLAG-tag insertion consisting of the eight aa-sequence, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, influenced the processing of bovine and human PrP^c in transfected BHK cells. HuPrP22FLAG was processed normally and expressed at high amounts similar to wild-type PrP on the cell surface of transfected cells. In contrast, introduction of the FLAG-tag close to the GPI-attachment site hampered the transport of huPrP227FLAG and boPrP239FLAG to the cell surface. Only low amounts of both proteins attained the cell surface. One possible explanation for this phenomenon might be the hampering effect of the FLAG-tag insertion close to the GPI-attachment site leading to a disruption of the transit of huPrP227FLAG and boPrP239FLAG to the cell surface and a retention of these proteins within cellular compartments of the secretory pathway such as the endoplasmic reticulum. However, glycosylation of all FLAG-tagged prion protein variants was not impaired. Therefore, recombinant glycosylated huPrP227FLAG and boPrP239FLAG have been purified from crude lysates by anti-FLAG-antibody chromatography. Such an epitope-tagged prion protein which can be purified simple and fast direct from total cell extracts might represent an useful tool to elucidate the physiological function of PrP^c and its role in the pathological mechanisms of TSEs.

Materials and methods

Cell culture

Baby hamster kidney cells (BHK-21 C13; ATCC CCL 10) were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin at 37 °C with 5 % CO_2.

Recombinant pSFV plasmid constructions

pSFV-1 (Liljestrom and Garoff, 1991), pSFV3-lacZ (Life Technologies) and the ORFs from human PrP and bovine PrP were used. The plasmid DNAs pSFV1-boPrP1-264, pSFV1-huPrP1-253 (Krasemann *et al*., 1996), pSFV1-huPrP-FFI (encoding aa 1-253 of the human PrP containing a FFI associated mutation at codon 178 Asp → Asn plus Met at position 129) and pSFV1-huPrP+9OR (encoding aa 1-253 of the human PrP including nine additional octapeptide repeats) (Krasemann *et al*., 1995) were used. pSFV1-huPrP22FLAG was

generated by the QuikChange™ site-directed mutagenesis method (Stratagene) employing pSFV1-huPrP1-253 DNA as a template. Construction of pSFV1-huPrP227FLAG (insertion of the FLAG-tag encoding sequence between codons 227 and 228 of the human PrP sequence) was described elsewhere (Hundt *et al*., in preparation). Construction of pSFV1-boPrP239FLAG. The insertion of a FLAG-tag encoding sequence between codon 239 and 240 of the bovine PrP sequence was performed by PCR using the pSFV1-boPrP1-264 plasmid DNA as a template. A 125 bp fragment (insertion of the FLAG encoding sequence) which encodes the carboxy-terminus of boPrP, was amplified introducing a *Hinf*I restriction site (endogenous site within codons 232 - 233) at the 5' end, the tag-encoding sequence between codon 239 and 240 as well as a *Sma*I site at the 3' end. This PCR-fragment encoding the C-terminal part of boPrP was digested with *Hinf*I and *Sma*I and ligated via the *Hinf*I restriction site to a 707 bp fragment encoding the aminoterminal part of boPrP from pSFV1-boPrP1-264 digested with *Hinf*I and *Sma*I. The ligated DNA fragments were cloned into the expression plasmid pSFV1 via the *Sma*I restriction sites resulting in pSFV1-boPrP1-239FLAG240-264. All constructs were confirmed by dideoxy sequencing.

SFV-mRNA generation by in vitro transcription

DNAs pSFV3-lacZ (Life Technologies), pSFV1-huPrP1-253, pSFV1-huPrP-FFI and pSFV1-huPrP+9OR, pSFV1-huPrP22FLAG, pSFV1-huPrP227FLAG, pSFV1-boPrP1-264 and pSFV1-boPrP239FLAG were linearized with *Spe*I following purification by phenol-chloroform extraction. 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 the transcripts was verified by agarose gel electrophoresis. RNA was stored at -20°C.

Cell transfection

For transfection, BHK cells were trypsinized, washed onces in PBS (w/o $MgCl_2$ and $CaCl_2$) and finally resuspended in PBS resulting in a cell number of $1x10^7$ cells per ml. 800 µl of the cell suspension were mixed with the individual SFV-RNA and then transferred to a 0.4 cm cuvette. Transfections were carried out by electroporation at room temperature by two consecutive pulses at 0,8 kV/25 µF using a Gene Pulser (BioRad). Cells were diluted in complete growth medium and plated on 10 cm cell culture dishes. For IF microscopy

maximal 1/10 volume of the electroporated cells ($8x10^5$) was diluted in complete growth medium and transferred onto 35 mm wells containing a sterile glass coverslip. Cells were incubated for at least 24 h. Transfection efficiencies as determined by transfecting SFV3-lacZ control RNA followed by X-gal staining were almost 100% for BHK cells.

Deglycosylation assay

$8x10^6$ BHK cells were transfected with recombinant SFV RNA as described above and plated on 10 cm culture dishes. 24 h post transfection cells were washed once with PBS, scraped off in PBS, harvested by centrifugation and finally lysed in 250 µl N-glycosidase F buffer by repeated freezing and thawing. The crude lysates were obtained by centrifugation at 14000 rpm 4°C for 15 min. A 20 µl aliquot of the supernatants were then treated with 2 units of N-glycosidase F (Roche Diagnostics) at 37 °C over night and analysed by western blotting.

Proteinase K digestion

$8x10^6$ BHK cells were transfected with recombinant SFV RNA as described above and plated on 10 cm culture dishes. 24 h post transfection cells were washed once with PBS, scraped off in PBS and harvested by centrifugation. Cells were resuspended in lysis buffer (10 mM Tris pH 7,5, 100 mM NaCl, 10 mM EDTA, 0,5% Triton-X100, 0,5% DOC) and finally lysed on ice for 15 min. The crude lysates were obtained by centrifugation at 14000 rpm 4°C for 15 min. proteinase K (Roche Diagnostics) was added to a 20 µl aliquot to get a final concentration of 2, 4 and 8 µg/ml. Reactions were carried out at 37 °C for 30 min, stopped with 0,5 mM pefabloc and analysed by western blotting.

Western blot analysis

After addition of Laemmli buffer, protein samples were separated on 12% SDS-polyacrylamide gels by SDS-PAGE and transferred to PVDF membranes Immobilon P (Millipore Corp.) for 1,5 h at 55 V. The membranes were blocked with I-Block (Tropix) in Tris-buffered saline pH 7,5 supplemented with 0,05% Tween, probed with the monoclonal anti-PrP antibodies 3B5 or 3F4 and thereafter with an appropiate peroxidase-coupled secondary antibody. The immunoreactivity was visualized by enhanced chemiluminescence (NEN™ Life Science Products) on Kodak BioMax MR-1 films and by staining with diaminobenzidine-tetrahydrochloride (Sigma).

Immunofluorescence analysis

To investigate the cellular localization of recombinant prion proteins in BHK cells, we used immunofluorescence microscopy. 24 hours post-transfectionally, cells were washed three times with PBS and fixed with 4% paraformaldehyde. Non-permeabilized cells were fixed with 2 % paraformaldehyde. After rinsing 3 times with PBS, cells were permeabilized for cytoplasmic staining with 0.2 % Triton X-100 (10 min/4 °C). The preparation was saturated with a 10 % FCS solution (in PBS) for 1 h at room temperature, washed and incubated with the primary antibody mAB 3B5 diluted in PBS with 10 % FCS for 1 h at room temperature. After washing three times with PBS the preparations were diluted in saturation buffer and incubated in the dark for 45-60 min with the secondary antibody (goat anti mouse) conjugated with indocarbocyanine (Cy3) (red). For nuclear staining 1 µg/ml 4′-6-diamidine-2-phenylindole (DAPI) for 10 min at RT was used. Please note that both the primary and secondary antibodies were added after fixing the cells. The coverslip was mounted with aqueous mounting medium (Fluoromount®) and the slides were examined using an axioviert fluorescence microscope (Zeiss) with appropiate filters. IF images were processed using Metamorph software®.

Purification of FLAG-tagged prion proteins by anti-FLAG antibody chromatography

Transfection of BHK cells with the rec. SFV-huPrP227FLAG or the rec. SFV-boPrP239FLAG RNA was performed as described above. 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. 48 h post transfection, cells were harvested, washed once with PBS and then lysed in PBS supplemented with 0.1% Triton-X100 by repeated freezing and thawing. The crude lysates were obtained by centrifugation at 14 000 rpm 4°C for 15 min. The FLAG-tagged proteins from the supernatants were bound over night to anti-FLAG M2 affinity gel (Sigma) by rotating at 4°C. Beads with immobilized protein were then washed four times with TBS, eluted over night by competition with 1 ml TBS containing 100 µg/ml FLAG peptides (Sigma) and dialyzed against 20 mM HEPES, pH 7.4. The purity and the concentration of the proteins were checked by SDS polyacrylamide gel electrophoresis followed by silver staining of the gel.

Antibodies

For western blot anaylses monoclonal anti-PrP antibodies 3F4 (Chemicon) and 3B5 (G. Hunsmann) were used. For IF analyses mAb 3B5 and a secondary Cy3 (indocarbocyanine)

conjugated antibody (used at 1:200 dilutions) (Jackson Laboratories and Southern Biotechnology, respectively) was used.

Table I. Summary of cellular localization and proteinase K status of rec. PrP mutants[a-g]

PrP mutants	Human Disease	Expression system	Cellular localization	Proteinase K status
MoPrP PG14 (+9OR)[a]	fCJD (model)	BHK cells transfected with Sindbis viral replicon[a]	Low level cell-surface expression[a]; retention in the endoplasmic reticulum[a]	resistant (up to 1 µg/ml PK 30 min 37°C)[a]
MoPrP PG11 (+6OR)[b,c,d]	fCJD (model)	Stably transfected CHO cells (pBC12/CMV)[b,c,d]	Cell-surface expression with additional mode of membrane association[b,c]	resistant (3,3 µg/ml PK 10 min 37°C)[b,d]
MoPrP E199K[b,d]	fCJD (model)	Stably transfected CHO cells (pBC12/CMV)[b,d]	Cell-surface expression with additional mode of membrane association[b]	resistant (3,3 µg/ml PK 10 min 37°C)[b,d]
MoPrP D177N/M128[a,b]	FFI (model)	Stably transfected CHO cells (pBC12/CMV)[a,b]	Low level cell-surface expression[a]; additional mode of membrane association[b]; retention in the endoplasmic reticulum[a]	resistant (3,3 µg/ml PK 10 min 37°C)[b]
MoPrP P101L[a,b]	GSS (model)	Stably transfected CHO cells (pBC12/CMV)[a,b]	Low level cell-surface expression[a]; additional mode of membrane association[b]; retention in the endoplasmic reticulum[a]	resistant (3,3 µg/ml PK 0 min 37°C)[b]
HaPrP+2/4/6OR[e]	fCJD (model)	Mouse fibroblast cells transf. with retroviral vector[e]	Aberrant cell-surface expression[e]	resistant (up to 0,8 µg/ml PK 10 min 37°C)[e]
HaPrP+4/6OR[e]	fCJD (model)	Mouse neuroblastoma cells transf. with retroviral vector[e]	Normal cell-surface expression[e]	resistant (up to 1 µg/ml PK 10 min 37°C)[e]
HuPrP D178N/V129[f]	fCJD	Human neuroblastoma cells transf. with episomal vector[f]	Impaired transport of unglycosylated PrP to the cell surface[f]	sensitive (at least 0.5 µg/ml PK 1 h or 5 µg/ml PK 5 min 37°C)[f]
HuPrP E200K[h]	fCJD	Human neuroblastoma cells transf. with episomal vector[h]	Underrepresentation of unglycosylated PrP at the cell surface[h]	resistant (3,3 µg/ml PK 10 min 37°C)[h]
HuPrP D178N/M129[f]	FFI	Human neuroblastoma cells transf. with episomal vector[f]	Impaired transport of unglycosylated PrP to the cell surface[f]	sensitive (at least 0.5 µg/ml PK 1 h or 5 µg/ml PK 5 min 37°C)[f]
HuPrP Q217R/V129[g]	GSS	Human neuroblastoma cells transf. with episomal vector[g]	Impaired transport to the cell surface[g]; accumulation of aggregated PrP in intracellular compartments[g]	resistant (3,3 µg/ml PK 5 min 37°C)[g]
HuPrP+9OR	fCJD	BHK cells transfected with Semliki Forest virus RNA	Expression at the cell surface and in intracellular compartments	resistant* (8 µg/ml PK 30 min 37°C)
HuPrP D178N/M129	FFI	BHK cells transfected with Semliki Forest virus RNA	Expression at the cell surface and in intracellular compartments	resistant* (8 µg/ml PK 30 min 37°C)

fCJD: familial Creutzfeldt-Jakob disease; FFI: Fatal Familial Insomnia; GSS: Gerstmann-Sträussler-Scheinker syndrome

MoPrP: mouse PrP; HaPrP: hamster PrP; HuPrP: human PrP; +OR: additional octarepeats; transf.: transfected; assoc.: association; PK: proteinase K

a (Ivanova *et al*., 2001) b (Lehmann and Harris, 1996a) c (Lehmann and Harris, 1995) d (Lehmann and Harris, 1996b) e (Priola and Chesebro, 1998) f (Petersen *et al*., 1996) g (Singh *et al*., 1997) h (Capellari *et al*., 2000)

*data described in this manuscript

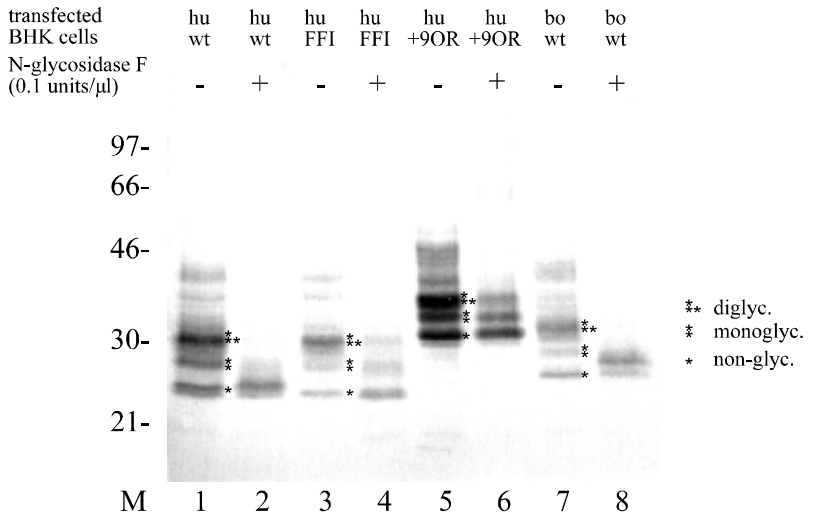

Fig. 1: Expression of wild-type as well as mutant human PrP and wild-type bovine PrP in transfected BHK cells and the effect of N-glycosidase F. Immunoblot analysis of prion proteins from total cell lysates of transfected BHK cells. Cells were transiently transfected with recombinant SFV RNAs encoding wild-type human PrP (lane 1), human PrP-FFI (lane 3), human PrP+9OR (lane 5) and wild-type bovine PrP (lane 7). 24 h p.t. the expression was analysed with mAb anti-PrP 3B5. Glycosylation patterns: di-, mono- and non-glycosylated PrP isoforms. Analysis of prion proteins huPrP (lane 2), huPrP-FFI (lane 4), huPrP+9OR (lane 6) and boPrP (lane 8) after N-glycosidase F treatment.

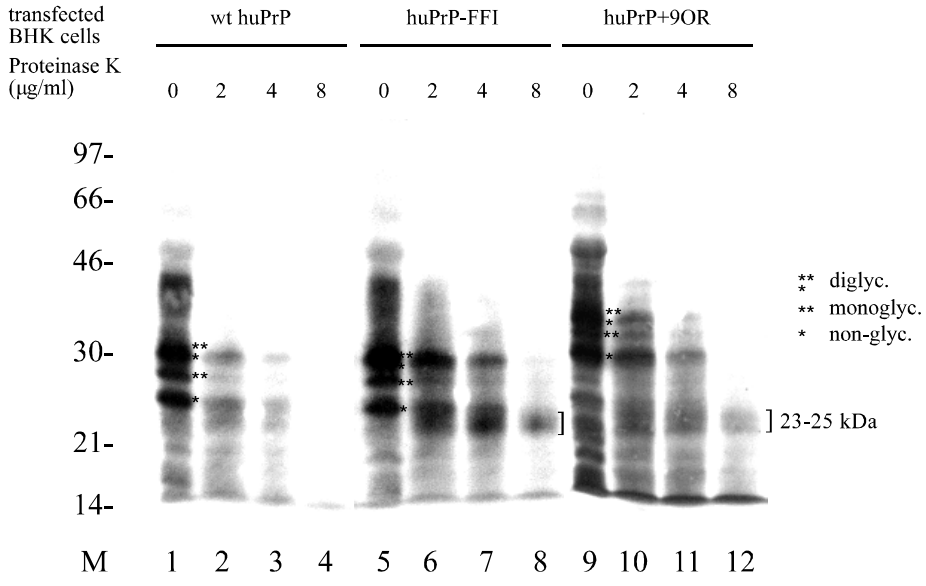

Fig. 2: Proteinase K status of prion protein mutants from total cell lysates of transfected BHK cells. Cells were transiently transfected with recombinant SFV RNAs encoding wild-type human PrP (lanes 1-4), human PrP-FFI (lanes 5-8) and human PrP+9OR (lanes 9-12). 24 h p.t. the expression was analysed by western blotting using mAb anti-PrP 3F4. Crude lysates were treated with increasing amounts of PK for 30 min at 37 °C: 0 µg/ml (lanes 1, 5 and 9), 2

µg/ml (lanes 2, 6 and 10), 4 µg/ml (lanes 3, 7 and 11) and 8 µg/ml (lanes 4, 8 and 12). PK resistant bands range fom 23 to 25 kDa as indicated (brackets).

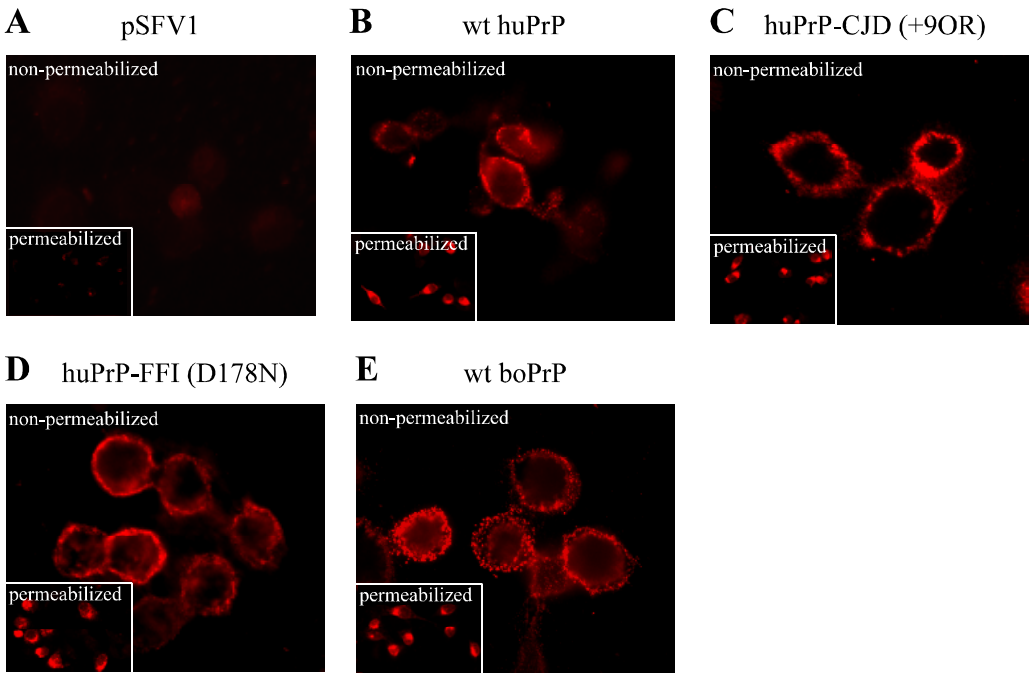

Fig. 3: Localization of huPrP, huPrP-FFI, huPrP-9OR and boPrP at the surface and in intracellular compartments of BHK cells transfected with rec. SFV RNAs. Subcellular location was determined by IF using the mAb anti-PrP 3B5 and a secondary Ab (goat anti-mouse IgG) konjugated with indocarbocyanine (Cy3). Cell surface immunostaining of non-permeabilized BHK cells transfected with (**A**) SFV-1 RNA (without any insert), (**B**) rec. SFV-huPrP1-253 RNA, (**C**) rec. SFV-huPrP-FFI RNA, (**D**) rec. SFV-huPrP+90R RNA and (**E**) rec. SFV-boPrP1-264 RNA. Insets A-E: Intracellular immunostaining of permeabilized, transfected BHK cells. (magnification A-E: x630).

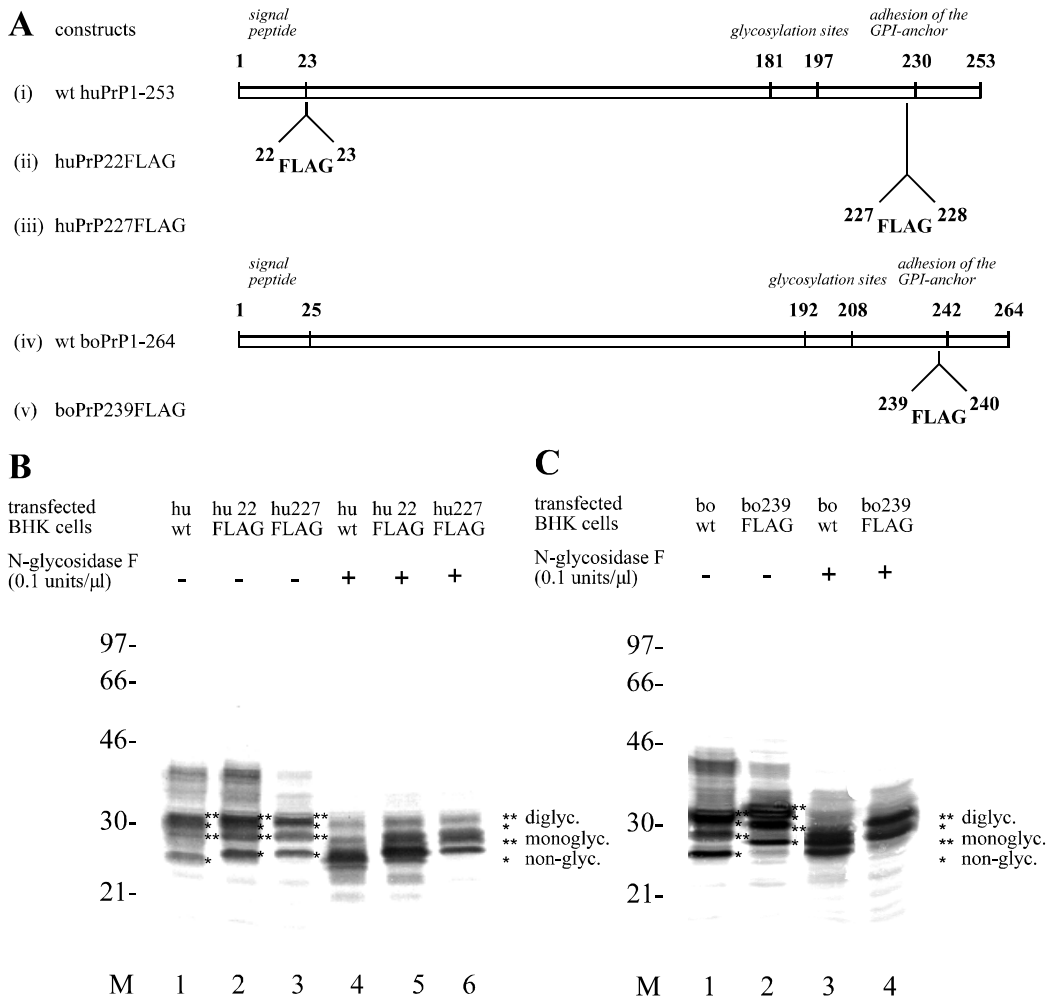

Fig. 4: Expression of glycosylated FLAG-tagged human and bovine PrP in BHK cells transfected with rec. SFV RNAs (**A**) Constructs of human PrP containing a FLAG-tag insertion either between aa 227 and 228 close to the GPI-anchor adhesion site or behind the N-terminal signal peptide between aa 22 and 23 and a bovine PrP construct with the FLAG-tag inserted between aa 239 and 240. (**B**) Expression and deglycosylation of wild-type and FLAG-tagged human PrP. (**C**) Expression and deglycosylation of wild-type and FLAG-tagged bovine PrP. Total cell extracts from SFV-transfected BHK cells were incubated over night with N-glycosidase F at 37 °C and analysed by western blotting. For detection mAb anti-PrP 3B5 was used. FLAG-tagged prion proteins were glycosylated in the same manner as wild-type PrP (B, lane 2 and 3 and C, lane 2) . After treatment with N-glycosidase F the glycosylated forms are clearly reduced accompanied by an increase of the non-glycosylated forms (B, lane 4, 5 and 6; C, lane 3 and 4). The shift in the molecular weight of non-glycosylated FLAG-tagged PrPs is due to the additional eight amino acids of the FLAG-tag.

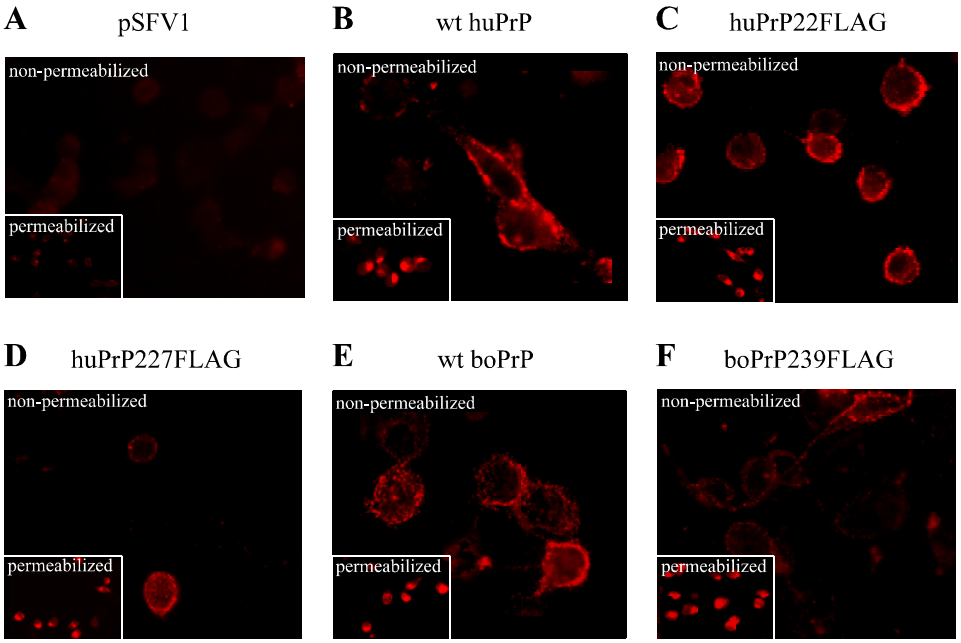

Fig. 5: Subcellular localization of wild-type and FLAG-tagged human and bovinePrP in BHK cells transfected with rec. Semliki Forest virus RNAs. Subcellular location was determined by IF employing the mAb anti-PrP 3B5 and a secondary Ab (goat anti-mouse IgG) konjugated with indocarbocyanine (Cy3). Immunostaining of non-permeabilized BHK cells transfected with (**A**) SFV-1 RNA (without any insert), (**B**) rec. SFV-huPrP1-253 RNA, (**C**) rec. SFV-huPrP22FLAG RNA, (**D**) rec. SFV-huPrP227FLAG RNA, (**E**) rec. SFV-boPrP1-264 RNA and (**F**) rec. SFV-boPrP239FLAG RNA. Insets A-F: Intracellular immunostaining of permeabilized, transfected BHK cells, respectively. (magnification A-F: x630).

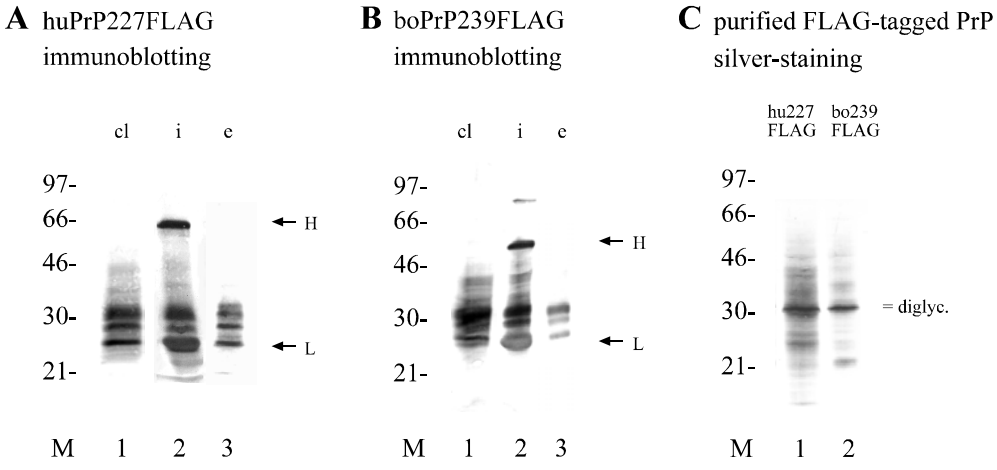

Fig. 6: Anti-FLAG-antibody purification of FLAG-tagged prion proteins from BHK cells. (**A** and **B**) Total cell extracts from transfected BHK cells expressing huPrP227FLAG (A) or boPrP239FLAG (B) were analysed by western blotting employing mAb anti-PrP 3B5. In order to purify FLAG-tagged PrP, crude lysates (cl) (A and B, lanes 1) were immobilized to

anti-FLAG M2 affinity gel (i) (A, B, lanes 2) and eluted by competition with FLAG peptides (e) (A and B, lanes 3). The arrows indicate the heavy and the light chain of the mAb anti-FLAG M2 (A and B, lanes 2). (**C**) The silver stained gel shows purified glycosylated huPrP227FLAG and boPrP239FLAG.

CHAPTER VI

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

submitted for publication to *Biol. Chem.* as:
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Intra- and interspecies interactions of prion proteins and effects of mutations and polymorphisms.

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.

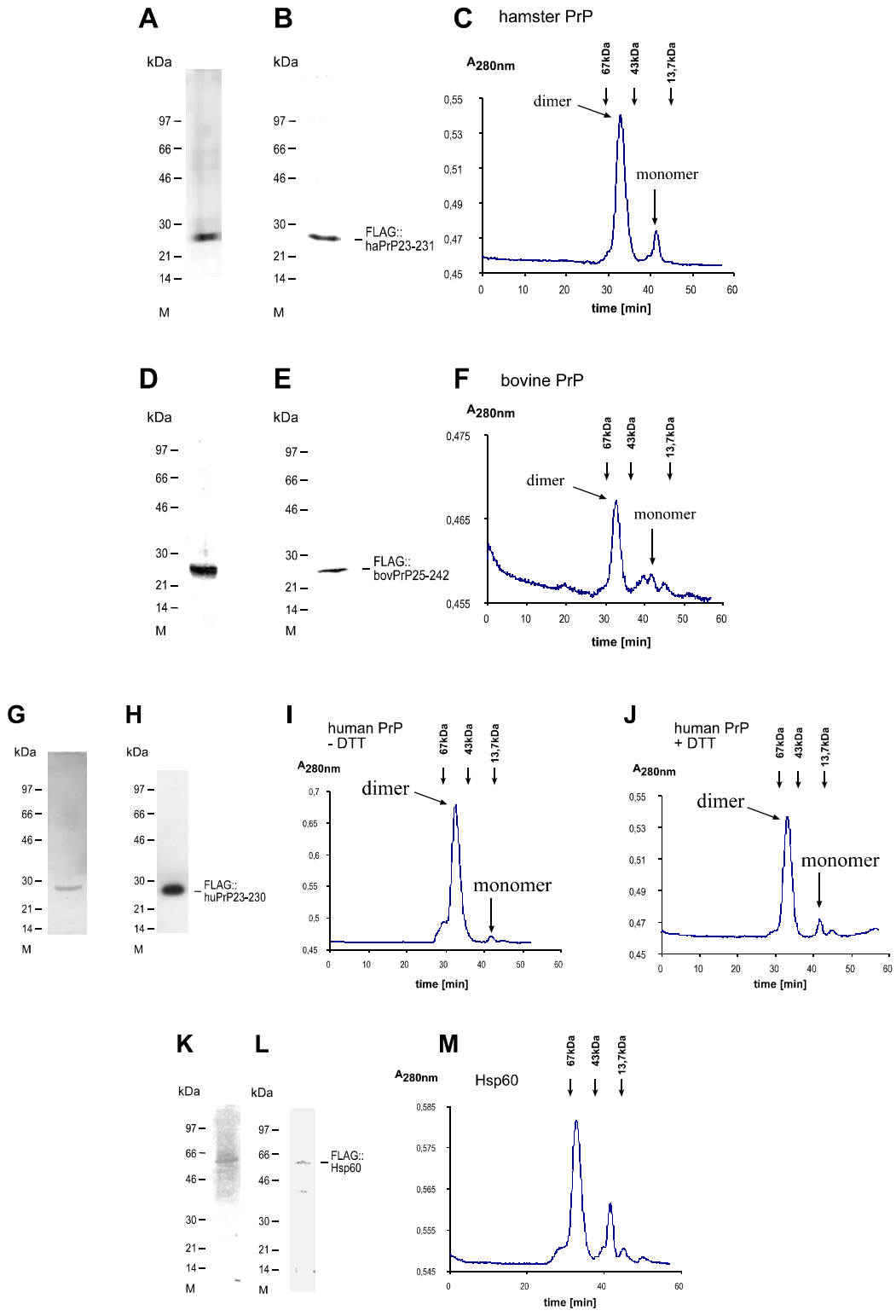

Fig. 1. Analysis of recombinant FLAG::PrP from hamster, cattle and man and FLAG::Hsp60 under native and denaturing conditions. 400 ng of purified FLAG::haPrP23-231 were analyzed by SDS-PAGE (12.5 % PA) followed by silver staining of the gel (**A**) and Western Blotting (**B**) employing the monoclonal 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.

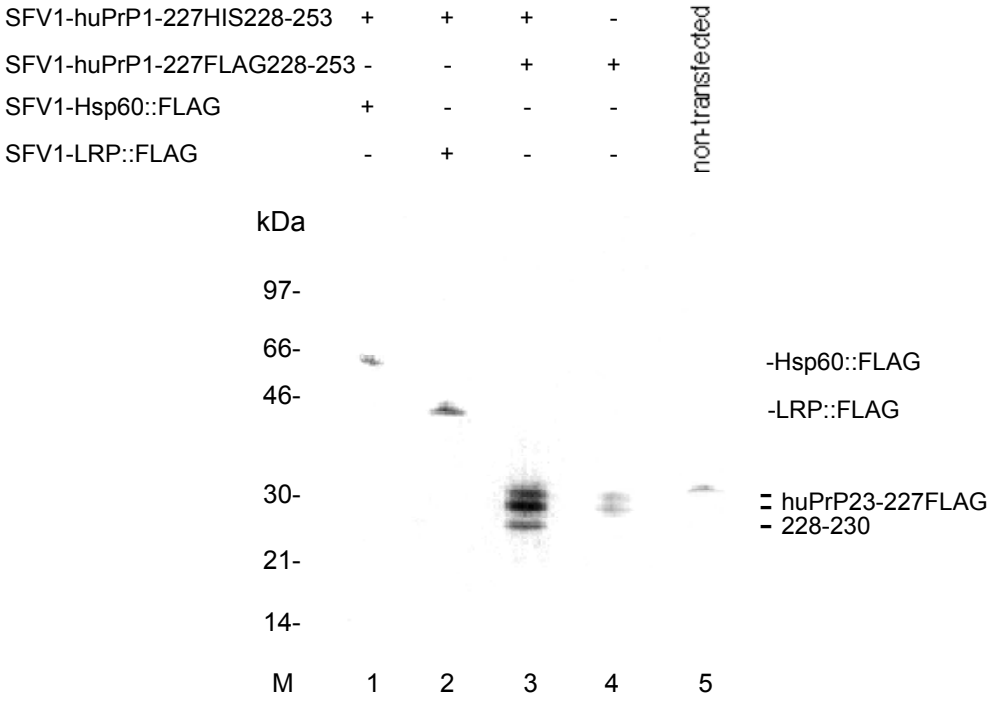

Fig. 2. Analysis of PrP/PrP interactions in BHK cells co-transfected with recombinant Semliki Forest virus RNAs. Extracts of total protein from BHK cells either non-transfected (lane 5), transfected with SVF1-huPrP1-227FLAG228-253 (lane 4) or co-transfected with SVF1-huPrP1-227FLAG228-253 and SVF1-huPrP1-227HIS228-253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

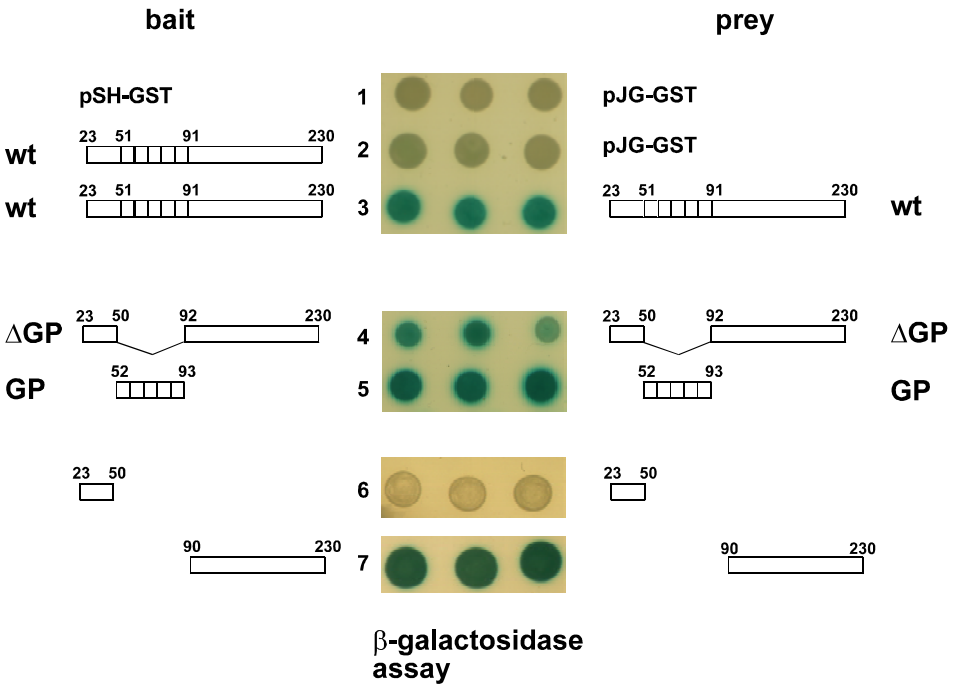

Fig. 3. Identification of PrP/PrP interaction domains in the yeast two-hybrid system (*S. cerevisiae*). Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (row 1 and 2), pJG-GST::PrP23-230 (row 3), pJG-GST::PrPΔGP (row 4), pJG-GST::GP52-93 (row 5), pJG-GST::PrP23-50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

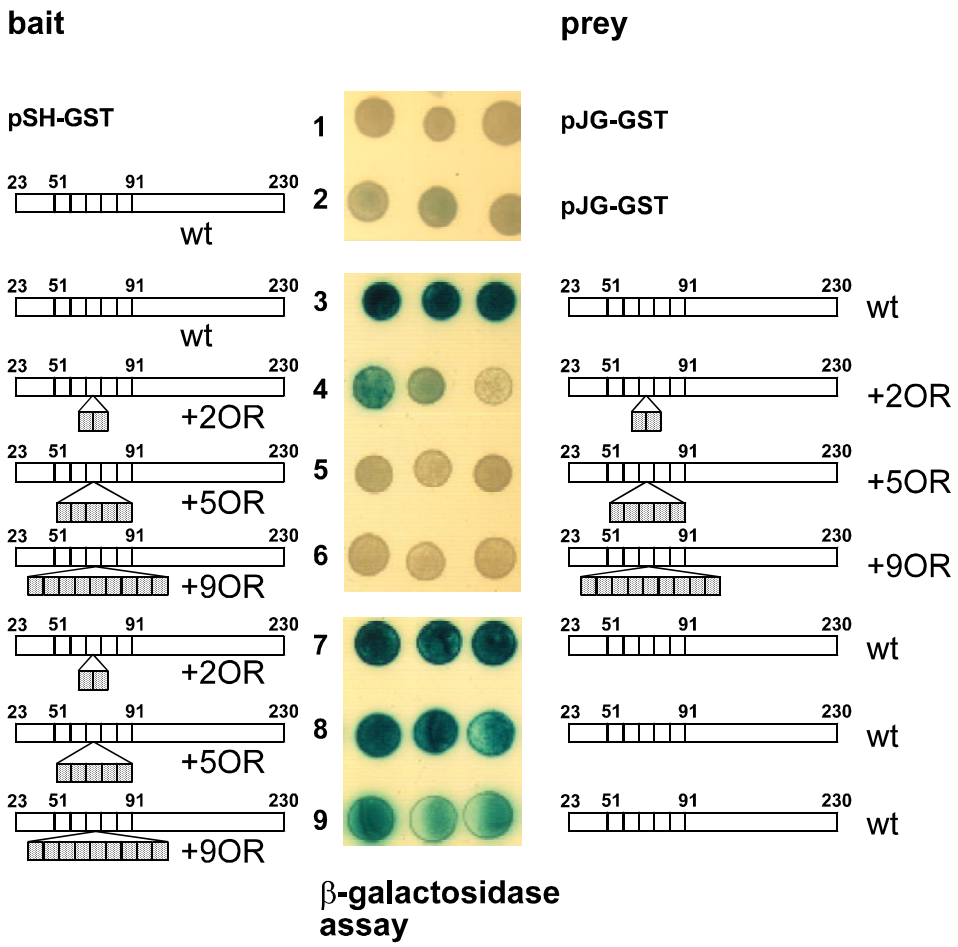

Fig. 4. Influence of additional octarepeats on the PrP/PrP interaction behaviour. Yeast cells encompassing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230 (rows 3, 7, 8 and 9), pJG-GST::PrP+2OR (row 4), pJG-GST::PrP+5OR (row 5), pJG-GST::PrP+9OR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

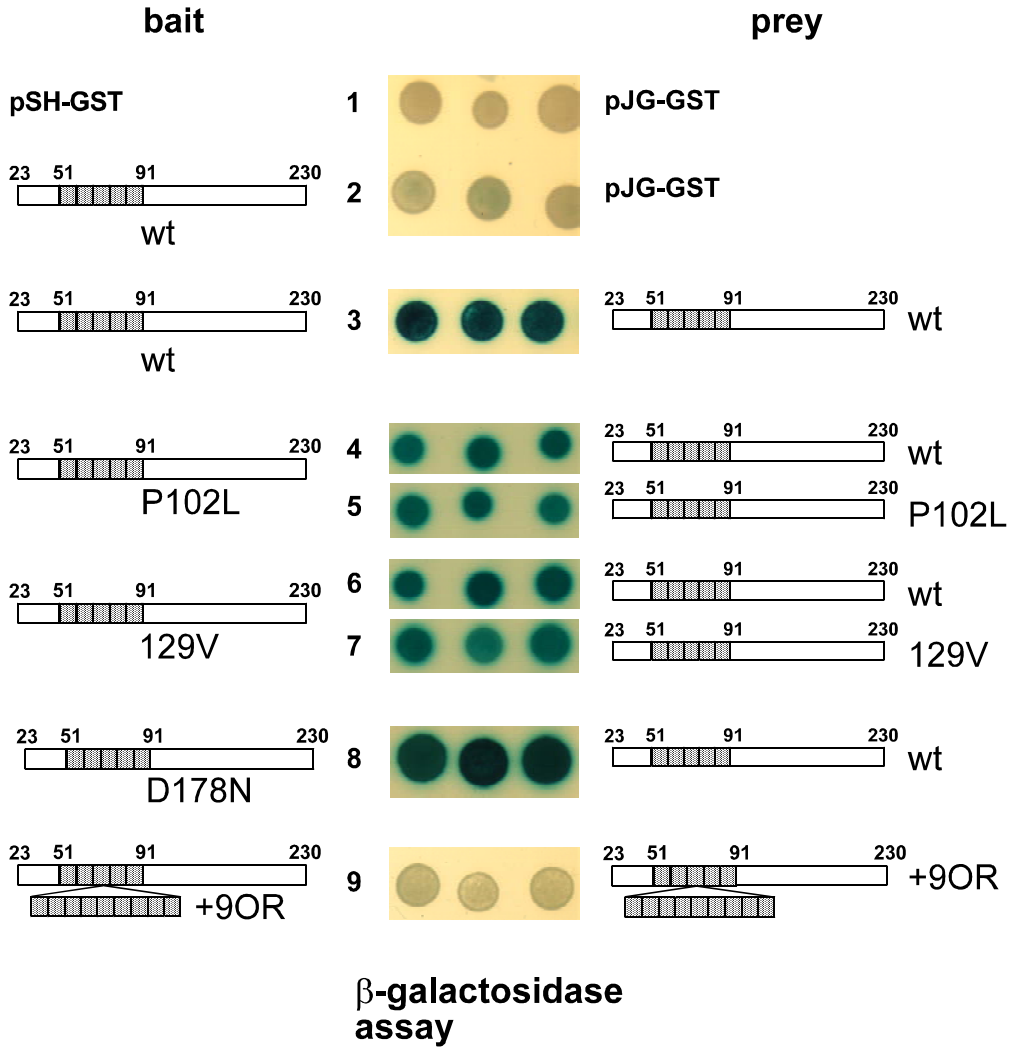

Fig. 5. Influence of the GSS-mutation P102L, the polymorphism M129V and the mutation D178N on the PrP/PrP interaction behaviour analyzed in the yeast two-hybrid system. Yeast cells containing the reporter plasmid pSH18-34 were co-transformed with prey plasmids pJG-GST (rows 1 and 2), pJG-GST::PrP23-230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

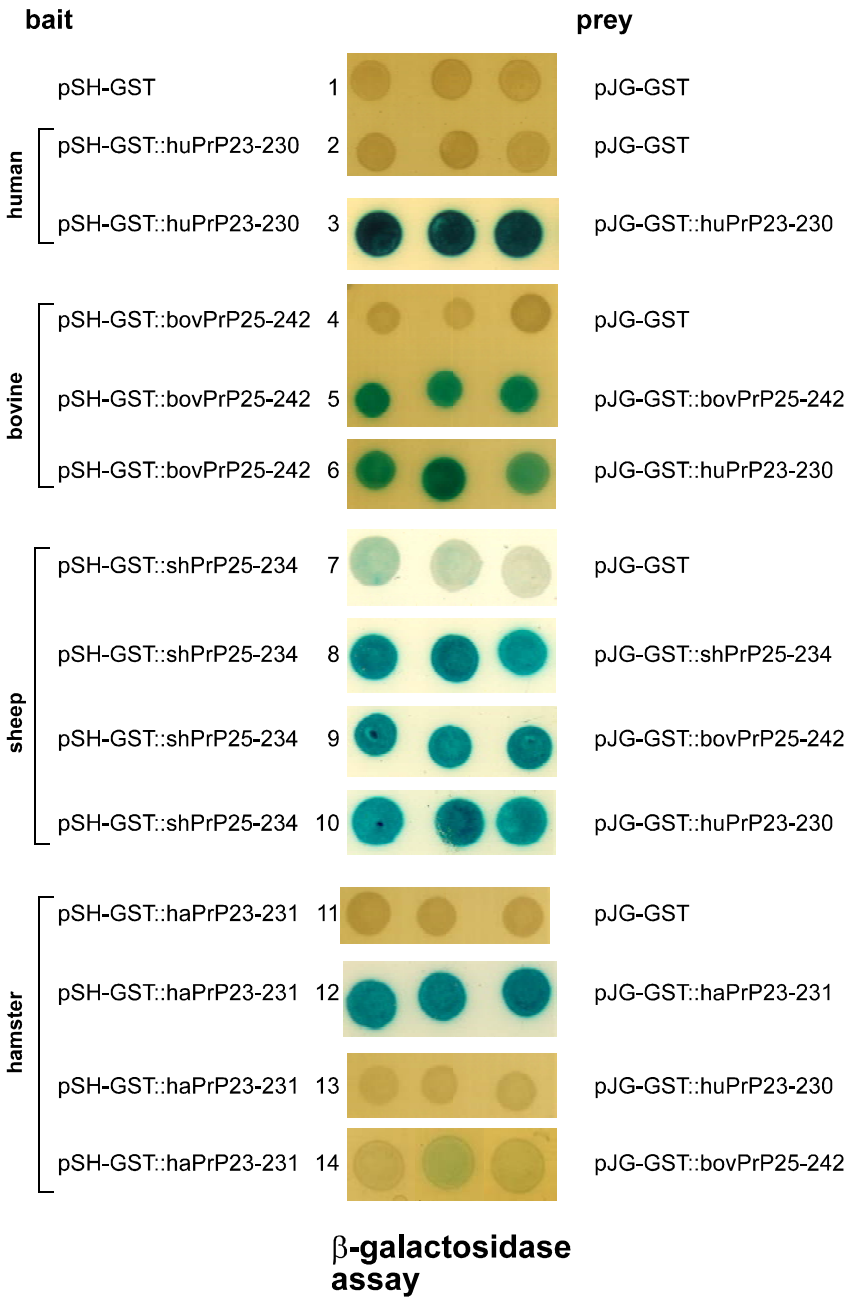

Fig. 6. Interaction analysis of PrP of different species in the yeast-two-hybrid system. The bait plasmids pSH-GST (row 1), pSH-GST::huPrP23-230 (rows 2 and 3), pSH-GST::bovPrP25-242 (rows 4-6), pSH-GST::shPrP25-234 (AQ) (rows 7-10) and pSH-GST::haPrP (rows 11-14) were co-transformed with the reporter-plasmid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

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Abbreviations

aa	amino acid
Ab	antibody
Ach(R)	acetylcholine (receptor)
AD	Alzheimer's disease
AmB	amphotericin B
APC	antigen presenting cell
APP	amyloid precursor protein
APS	ammonium persulfate
ATP	adenosine triphosphate
BD	binding domain
BHK	baby hamster kidney
boPrP	bovine PrP
bp	base pair
BSA	bovine serum albumine
BSE	bovine spongiforme encephalopathy
CD	circular dichroism
cDNA	complementary (to mRNA) DNA
CFTR	cystic fibrosis transmembrane regulator
CHO	chinese hamster ovary
CJD	Creutzfeldt-Jakob disease
CLDs	caveolae-like domains
CWD	chronic wasting disease
Cy2	carbocyanine
Cy3	indocarbocyanine
DAPI	4′-6-diamidine-2-phenylindole
DS-500	dextran sulfate 500
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dpl	doppel
ECM	extracellular matrix
e.g.	for example [Lat.: exempli gratia]
EHS	Engelbreth-Holm-Swarm
ER	endoplasmic reticulum
FACscans	Fluorescence-activated cell scans
FCS	fetal calf serum
FFI	fatal familial insomnia
FGF(R)	fibroblast growth factor (receptor)
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
HPA-23	heteropolyanion 23
Hsp	heat shock protein
HSPG	heparan sulfate proteoglycan

huPrP	human PrP
IDX	4‘-iodo-4‘-deoxy-doxorubicin
i.e.	that is [Lat.: id est]
IF	immunofluorescence
kb	kilo bases
kDa	kilo Dalton
LR	laminin receptor
LRP	laminin receptor precursor
LRS	lymphoreticular system
mAb	monoclonal Ab
MAFF	ministery of agriculture, fisheries and food
min	minutes
moPrP	mouse PrP
mRNA	messenger RNA
M	molar
MHC	major histocompatibility complex
MW	molecular weight
N2a	murine neuroblastoma cells
NMR	nuclear magnetic resonance
NT2	human teratocarcinoma cells
PAA	polyacrylamide
pAb	polyclonal Ab
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPLC	phosphatidylinositol-specific phospholipase C
PK	proteinase K
PrP^{c}	cellular prion protein
PrP-CAA	prion protein cerebral amyloid angiopathy
Pli	PrP ligand
PrP^{Sc}	prion scrapie (pathogenic isoform of PrP^{c})
rec.	recombinant
rER	rough endoplasmic reticulum
RNA	ribonucleic acid
rpm	rotations per minute
SDS	sodium dodecyl sulfate
SFV	Semliki Forest virus
SP54	pentosan polysulfate
TEMED	N,N,N',N'-tetramethylethylendiamine
TGN	trans-Golgi network
TMAO	trimethylamine-*N*-oxide
TMD	transmembrane domain
TNF(R)	tumor necrosis factor (receptor)
TSE	transmissible spongiforme encephalopathy
UK	United Kingdom
vCJD	(new) variant CJD
VEE	Venezuelan equine encephalitis virus
VLA-6	very late activation antigen-6
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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