Dissertation zur Erlangung des Doktorgrades der Fakultät für Chemie und Pharmazie der Ludwig-Maximilians-Universität München

The role of the 37-kDa/67-kDa laminin receptor in the cellular metabolism of the prion protein

Christoph Leucht
aus
Frankfurt am Main

2003

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Priv.-Doz. Dr. Stefan Weiß betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 10.01.2003

Christoph Leucht

Dissertation eingereicht am 10.01.2003

1. Gutachter: Priv.-Doz. Dr. Stefan Weiß
2. Gutachter: Prof. Dr. Karl-Klaus Conzelmann

Mündliche Prüfung am 11.02.2003

DANKSAGUNG

Ich danke Herrn Priv.-Doz. Dr. Stefan Weiß für die Betreuung der Doktorarbeit, der Erstellung des Erstgutachtens und für den schier unerschöpflichen Einsatz die Forschung und die Publikationen voranzutreiben. Dank auch für diverse Besuche internationaler Kongresse.

Herrn Prof. Dr. Klaus Conzelmann danke ich für die Erstellung des Zweitgutachtens.

Mein Dank gilt auch Herrn Prof. Dr. Rudi Grosschedl, der das Genzentrum mit sicherer Hand zu neuen, interessanten Ufern führt.

Ohne die Vorarbeit von Prof. Dr. Ernst-Ludwig Winnacker wäre es wohl nicht möglich gewesen meine Doktorarbeit am Genzentrum anzufertigen, da das Genzentrum wohl nicht existierte. Hierfür mein Dank.

Ich bedanke mich bei allen aktuellen und ehemaligen Mitarbeitern der Arbeitsgruppe Weiß für tatkräftige Unterstützung. Insbesondere danke ich Martina Brecelj dafür, daß sie ihre Diplomarbeit in der Arbeitsgruppe Weiß angefertigt hat, weil sie mein Leben sonst nicht in einer wunderbaren Weise hätte beeinflussen können. Außerdem wäre es sonst nie zu einer unglaublich “fruchtbaren” Beziehung gekommen.
Mein dank gilt auch allen Kollegen im Genzentrum, die mich während meiner Zeit als Doktorand unterstützt haben.

Ich danke den Rödelheimer Jungs (inkl. Scheich) für ihre erfrischende Art und unzählige Festivitäten, die mich mental sehr viel weiter gebracht haben.

Ein ganz besonderes Dankeschön geht an meine Eltern, die mich kontinuierlich unterstützt haben und ohne die ein Studium oder gar eine Promotion sehr, sehr schwer gewesen wäre.

Die vorliegende Arbeit wurde in der Zeit von Januar 1999 bis Dezember 2002 im Labor von Priv.-Doz. Dr. Stefan Weiß am Genzentrum der Ludwig-Maximilians-Universität München angefertigt.

Im Verlauf dieser Arbeit wurden folgende Veröffentlichungen publiziert:

Leucht, C., Simoneau, S., Rey, C., Rieger, R., Lasmézas, C.I. and Weiss, S. (2003) The 37kDa/67kDa laminin receptor is required for PrP^{Sc} propagation in scrapie-infected neuronal cells. *EMBO Reports*, **4**, 290–295.

Simoneau, S., Haïk, S., **Leucht, C.**, Dormont, D., Deslys, J.-P., Weiss, S. and Lasmézas, C.I. (2003) Different Isoforms of the Non-Integrin Laminin Receptor are Present in Mouse Brain and Bind PrP. *Biol. Chem.*, **384**, 243–246

Hundt, C., Gauczynski, S., **Leucht, C.**, Riley, M.-L. and Weiss, S. (accepted) Intra- and interspecies interactions of prion proteins and effects of mutations and polymorphisms. *Biol. Chem.*

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

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

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

Gauczynski, S., Peyrin, J.M., Haik, S., **Leucht, C.**, Hundt, C., Rieger, R., Krasemann, S., Deslys, J.P., Dormont, D., Lasmezas, C.I. and Weiss, S. (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J.*, **20**, 5863-5875.

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

Summary

Prion diseases are fatal neurodegenerative diseases which occur in mammals. The abnormal form (PrP^{Sc}) of the host encoded prion protein (PrP^{C}) is the main player in the pathogenesis of prion diseases. However, the pathogenesis of the disease and the cellular function of the prion protein is not fully understood.
Both PrP^{C} and PrP^{Sc} are thought to interact with different molecules during their cellular metabolism such as the 37kDa laminin receptor precursor (LRP) which was identified as an interactor of PrP^{C} in a yeast two-hybrid screen.

Here, the influence of the 37kDa LRP and its mature form, the 67kDa laminin receptor (LR) on the cellular fate of PrP^{C} and PrP^{Sc} has been investigated.
PrP^{C} is found on the surface of neuronal and non-neuronal cells. The same is true for the 37-kDa/67-kDa laminin receptor (LRP/LR) as shown by flow cytometry, immunofluorescence, and Western blot analysis of purified plasma membranes of N2a cells. Both the 37kDa- and the 67kDa-form have been found in purified plasma membrane fractions of N2a cells.
Binding of externally added recombinant PrP^{C} to N2a cells has been shown to be dependent on the availability of LRP/LR on the cell surface. Blocking of LRP/LR with specific antibodies resulted in a total inhibition of the binding. The internalization of PrP^{C} has also been shown to be LRP/LR-dependent, which has been shown by trypsin treatment of the cells. By lowering the incubation temperature from 37°C to 4°C, the internalization of PrP^{C} was totally abolished, indicating that the process is active and receptor mediated. In summary these data demonstrate that the 37kDa/67kDa LRP/LR acts as the cell surface receptor for the cellular form of the prion protein.

Scrapie infected neuronal cells are a well known model system for the pathogenesis of prion diseases. They can be used to test the ability of different substances and drugs to inhibit the formation of abnormal prion protein (PrPres) in these cells.
The incubation of the cells with the LRP/LR specific antibody W3 resulted in a repression of PrPres formation. Furthermore, transfection of these cells with (i) a plasmid encoding for an antisense-LRP RNA and (ii) small interfering RNAs (siRNAs) specific for the LRP cDNA sequence transiently reduced the LRP/LR and the PrP^{C} levels in these cells and subsequently

blocked PrPres formation permanently. These experiments showed that ablation of LRP/LR does not only affect the PrP^{C}- but also the PrP^{Sc}-metabolism and that LRP/LR is required for PrPres propagation in cultured cells.

Different isoforms of the 37-kDa/67-kDa laminin receptor have been reported. In addition to the 37kDa precursor form and the 67kDa mature form two other isoforms of 60kDa and 220kDa respectively, have been identified in mouse brain. All four isoforms bound PrP in overlay assays using either radiolabelled or immunodetected recombinant PrP.

High-level expression of glycosylated PrP is of particular interest to investigate PrP structure and function. Dimers of the prion protein have been shown to exist in a pre-oligomerization state of the infectious agent. A crystal structure of a fully glycosylated PrP-dimer might clarify the conversion process.
The methylotrophic yeast *Pichia pastoris* has been used to express and partially purify a glycosylated monomer and a covalently linked dimer of the human prion protein. Both proteins revealed proteinase K sensitivity and, as shown by plasma membrane purification, were found in the plasma membrane of the yeast. The proteins might act as tools for crystallization trials and PrP^{C} PrP^{Sc} conversion studies.

CHAPTER I

INTRODUCTION

1 Prion diseases

Transmissible spongiform encephalopathies (TSEs), are neurodegenerative diseases caused by prions (*proteinaceous infectious particles*) which have been observed in humans and animals. Human TSEs are Creutzfeldt-Jakob-Disease (CJD), fatal familiar insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Kuru, whereas bovine spongiform encephalopathy (BSE) is to be found in cattle, chronic wasting disease (CWD) in elk and deer and scrapie in sheep and goat (Table I) (Lasmézas and Weiss, 2000; Weissmann and Aguzzi, 1997). The infectious agent, termed prion, is thought to consist of an abnormal protein as the sole infectious component. Lacking any nucleic acid, these diseases represent not viral diseases but an entirely new class of infectious diseases, termed prion diseases (Prusiner, 1982). The abnormal, infectious protein causing TSEs is termed PrP^{Sc} (the scrapie form of the prion protein) and represents an isoform of the host-encoded and naturally expressed protein PrP^{C} (the cellular form of the prion protein) (Prusiner, 1998). PrP^{Sc} and its proteinase K resistant form PrP 27-30, which is 142 amino acids in length (in case of hamster PrP), are present predominantly in brains or lymphoreticular organs of humans or animals suffering from prion diseases and give rise to amyloid plaques (McKinley *et al.*, 1991). PrP^{C} and PrP^{Sc} have the same primary structure, but differ in their three dimensional structure. The key event in pathogenesis of prion diseases is the conversion from the cellular homolog to the disease-causing form. It is widely believed, that this process does not involve any nucleic acid and resulting from this, Stanley B. Prusiner phrased the "protein-only" hypothesis first in 1982 (Prusiner, 1982). PrP^{C} has a high content in α-helix (42%) and a low β-sheet content (3%), whereas PrP^{Sc} has less α-helix and more β-sheet structures (45%) (Caughey *et al.*, 1991; Pan *et al.*, 1993).

1.1 The 'prion' – an extraordinary infectious agent

Prusiner was not the first to propose that an abnormal protein might be responsible for TSEs. The idea was suggested first in 1966 by Tikvah Alper (Medical Research Council, Hammersmith Hospital, London). He found that ultraviolet radiation, that is commonly used

to inactivate nucleic acid, does not abolish scrapie infectivity (Alper *et al.*, 1967; Alper *et al.*, 1966). In 1967, J. S. Griffith (Bedford College, London) proposed that infectivity in scrapie might be caused by a normal cellular protein that displays an altered conformation (Griffith, 1967). Fifteen years later Stanley B. Prusiner took up and developed these ideas and conducted convincing biochemical analyses on the infectious agent. He purified the prion protein (PrP) from the infectious material that he termed 'prion' and showed that PrP is the major component of the infectious agent (Prusiner, 1982; Prusiner *et al.*, 1982; Prusiner *et al.*, 1981). A few years later, the prion protein was sequenced and cloned in collaboration with Charles Weissmannn (Basler *et al.*, 1986; Oesch *et al.*, 1985).

1.2 The history of prion diseases

In contrast to the prion hypothesis, prion diseases have a much longer history. In 1732 the first report of natural sheep scrapie emerged in the UK (for review see (Schreuder, 1994)). It owes its descriptive name to the phenomenon that in some cases the infected sheep scrape off their wool due to intense itching. The first report of a human TSE appeared in 1920. Hans Gerhard Creutzfeldt described a case of progressive and fatal disease in a 23-year-old woman, who presented with mental and neurological disorders (Creutzfeldt, 1920). One year later Alfons Maria Jakob described the symptoms of three other individuals suffering from a disease in the pyramidal and extrapyramidal motor systems (Jakob, 1921). In 1922 the term 'Creutzfeldt-Jakob disease' was introduced by Spielmeyer to describe some cases of neurodegenerative diseases that were characterized by the loss of neurons and gliosis (for review see (Kretzschmar, 1993)). At that time the agent responsible for the disease was unknown. Some decades later Gajdusek and Zigas reported a transmissible disease termed 'kuru' that affected children and mostly female adults in the Eastern highlands of Papua New Guinea (Gajdusek and Zigas, 1957; Zigas and Gajdusek, 1957). In 1959, Hadlow observed neuropathological similarities between kuru and scrapie and in the same year similarities between CJD and Kuru were observed, indicating a link between transmissibility and disease (Klatzo *et al.*, 1959). Indeed, it was found that all three diseases were transmissible. In 1961, Chandler transmitted scrapie to mice. In 1966, Kuru was transmitted to chimpanzees and in 1968 CJD was also

transmitted to chimpanzees (Chandler, 1961; Gajdusek *et al.*, 1966; Gibbs *et al.*, 1968). These findings demonstrated the transmissibility of the disease and the term 'transmissible spongiform encephalopathy' was introduced. Nevertheless, the nature of the agent was still unclear and it was widely believed that a 'slow virus' was responsible for the disease, as was suggested by the Icelandic virologist Björn Sigurdsson in 1954. He claimed that a slow virus has

- a very long incubation period
- a shorter progressive clinical course leading to death
- limitation of the infection to a single host and
- pathological changes in a single organ or tissue.

In 1976, Charlton Gajdusek was awarded the first Nobel prize for medicine within this scientific field for his work on 'slow virus' infections. About two decades later the second Nobel prize in this field, now known as the prion field, was awarded to Stanley Prusiner for his 'protein-only' hypothesis postulated in 1982. This award reflects the enormous work that has been done over the last twenty years by him and other scientists in the field to prove that a protein, the prion protein, is the major player in the pathogenesis of prion diseases. It is a matter of speculation whether Prusiner would have been given the award if there has not been a large-scale epidemic among cattle in the United Kingdom that provoked a large public outcry. In 1985, the first case of a novel spongiform encephalopathy in cattle appeared (Wells *et al.*, 1987). Since then more than 180,000 cattle have been affected by Bovine spongiform encephalopathy (BSE) in the UK and a few thousand in other European countries and Japan. As a consequence, several cases of spongiform encephalopathies in captive and zoo animals of many different species occurred (Schreuder, 1994). Most likely, the zoo animals had been fed with contaminated food products. The report of affected zoo animals and also domestic cats gave rise to considerable concern about the possibility or risk of transmission of BSE to man. To address this problem the National CJD Surveillance Unit was established in Edinburgh in 1990. In 1996, it had become evident that 10 persons were affected by a new form of CJD (vCJD) that is distinct from the classical, known forms of CJD (Will *et al.*, 1996). In contrast to the classical CJD cases all 10 patients were younger than 40 years of age

(Will *et al.*, 1996). Since then the number of vCJD cases has steadily increased, and by December 2002 a total number of 141 vCJD patients have been registered.

Table I Transmissible spongiform encephalopathies in animal and man

Man	Year (Country) of first report	Animal	Year (Country) of first report
Sporadic Creutzfeldt-Jakob disease (sCJD)	1920 (Germany)	Scrapie (sheep and goats)	1732 (UK)
Familial Creutzfeldt-Jakob disease (fCJD)	1924 (Germany)	Transmissible mink encephalopathy (TME) (farmed mink)	1947 (USA)
Gerstmann-Sträussler-Scheinker syndrome (GSS)	1928/1936 (Austria)	Chronic wasting disease (CWD) (captive mule deer and elk)	1980 (USA)
Kuru	1957 (New Guinea)	Bovine spongiform encephalopathy (BSE)	1986 (UK)
Iatrogenic Creutzfeldt-Jakob disease (iCJD)	1974 (USA)		
		Exotic ungulate encephalopathy (zoo animals)	1986 (UK)
Fatal Familial Insomnia (FFI)	1986 (Italy)		
		Feline spongiform encephalopathy (FSE) (captive/domestic members of the cat family)	1990 (UK)
Variant Creutzfeldt-Jakob disease (vCJD)	1996 (UK)		

1.3 Animal Diseases

Scrapie is a progressive and fatal neurological disease in sheep and very rarely in goats. It has been well known in Europe for more than two centuries and was also reported in other continents, such as North America. The transmissibility of scrapie from sheep to goat was first demonstrated in 1939 (Cuille and Chelle, 1939). Remarkably, it was never reported that the disease spread to humans (Schreuder, 1994).

Transmissible mink encephalopathy (TME) occurs endemically in ranch-ranged mink and has clinico-pathological features similar to those of scrapie. It was first reported in 1947 in Wisconsin, USA, and since then, more than 23 sporadic outbreaks have been reported worldwide, including the United States, Canada, Finland, Germany, and the republics of the former Soviet Union. The last outbreak was in the USA in 1985, after an outbreak-free period of 22 years (Schreuder, 1994).

Chronic wasting diseases (CWD) appeared for the first time in 1967 in captive mule deer in Colorado, USA, and was first reported in 1980 (Williams and Young, 1980). In 1982, it was found in captive Rocky Mountain elk. During the past years it was also found in free-ranging animals all over the Rocky Mountains from Canada to the USA (Schreuder, 1994). Very recently, one imported case of CWD in Korea appeared. The mule elk was imported into Korea from Canada on March 9, 1997 and represents the first case of CWD outside the U.S.A. and Canada (Sohn *et al.*, 2002).

The first case of *bovine spongiform encephalopathy* (BSE) occurred in 1985 and was diagnosed in 1986 (Wells *et al.*, 1987). More than 180,000 cases have been reported by December 2002. The epidemic was most likely caused by feeding meat and bone meal that was not properly inactivated to a large number of cattle. The source of the infectious agent is still speculative. The 'sheep-origin' hypothesis proposes a transmission of scrapie material from sheep to cattle (Wilesmith *et al.*, 1991), whereas the 'bovine-origin' hypothesis claims that the disease originated from a rare case of bovine prion disease and spread in cattle by ingestion of contaminated meat and bone meal (Philips, 2000).

Since 1986 several cases of *Exotic ungulate encephalopathy* occurred. These cases might be directly linked to the BSE epidemic since such cases were not reported previously. Captive antelope, greater kudu, gemsbok, eland, bison, nyala, Arabian oryx and scimitar-horned oryx were affected (Schreuder, 1994).

In 1990, the first case of feline spongiform encephalopathy (FSE) was reported in the UK. Since then, several cases of FSE in domestic cats, puma, cheetah, ocelot and tiger have occurred.

1.4 Human Diseases

1.4.1 Kuru

Kuru is a fatal neurodegenerative disease affecting a group of natives in Papua New Guinea called the 'Fore' people. It is characterized by progressive cerebella ataxia followed by dementia, leading to death in less than a year from onset of disease. However, one case of a more than 40-year-old man has been reported, suggesting that there might be extremely long incubation times (J. Collinge, personal communication). The disease was first described by Vincent Zigas, a physician working for the Australian Public Health Service, and Charlton Gajdusek, an American virologist and pediatrician of the US National Institutes of Health (Gajdusek and Zigas, 1957; Zigas and Gajdusek, 1957). The mysterious disorder was described with the term 'Kuru' meaning 'shivering'. It originated probably in the northern part of the Fore district at the beginning of the last century and spread through 169 villages and hamlets in the district and to neighbouring linguistic groups that intermarried with the Fore people. In the late 1950's the average incidence of kuru in the Fore district was 1% but in some villages there was a much higher incidence rate of ≥10% (Gajdusek and Zigas, 1959). Between 1957 and 1982 more than 2,500 people died of Kuru, the youngest patient being 5 years old. Most of the patients were adult women (67%), followed by children (23%) and

Table II Codon 129 polymorphism

Codon 129	Normal population	Sporadic CJD	New variant CJD
MM	39%	80%	100%
MV	50%	8%	--
VV	11%	12%	--

adult men (11%). This phenomenon can be explained by the mourning ritual that was practised among the Fore people. Mostly women and smaller children participated in the ritual, whereas men seldom ate the brain and meat of the dead woman. In the late fifties the ritual practice was stopped and today only a few cases of Kuru are seen every year and there are no patients born after 1959 (Gajdusek, 1996). The codon 129 genotype found in Kuru patients has been matched with age and duration of illness. It was found that methionine homozygosity was overrepresented in younger patients, in patients with a short incubation period and in patients with a short duration of disease. No influence on clinical symptoms was observed (Cervenakova *et al.*, 1998).

1.4.2 Sporadic Creutzfeldt-Jakob disease (sCJD)

Sporadic Creutzfeldt-Jakob disease is a rare disease, with an incidence of only 0.5-2 affected patients per 1.000.000 people per year. The clinical course is generally rapid and in most cases the duration is less than 12 months with a mean duration of 4 months (Wells *et al.*, 1987). The disease is reported in all age groups, but the median age of onset is the seventh decade. It very rarely occurs in people younger than 40 years and men and women are equally affected. Due to the age distribution the incidence of sCJD is 3 per million per year in the 65 to 74-year-old group and only 0.2 per million in the population below 40 years of age (Collinge and Palmer, 1992; Ironside, 1998). The sporadic form accounts for ~85% of all CJD cases, whereas ~15% occur in a familial context. Explanations for the etiology of sCJD include two hypotheses,

- the occurrence of age-related somatic mutations that happen randomly and might result in a facilitated conversion of PrP^{C} to PrP^{Sc} or
- the spontaneous conversion of PrP^{C} to PrP^{Sc} without involvement of mutations (Prusiner *et al.*, 1998).

Very recent findings try to explain sporadic Creutzfeldt-Jakob disease by suggesting a conversion of PrP in the cytosol where misfolded PrP^{C} is transported from the endoplasmatic reticulum. There, it might convert in a rare event to PrP^{Sc} representing a seed for further aggregation (Ma and Lindquist, 2002).

A common methionine/valin polymorphism is of particular interest in both sporadic CJD and variant CJD (see chap. 1.4.4), with either methionine or valin present at residue 129 of the human prion protein. In Caucasians the general distribution is 38% homozygosity for methionine, the most frequent allele, 51% heterozygosity and 11% of the population is homozygous for valine (Owen *et al.*, 1990) (Table II). In sporadic CJD more than 80% of the patients are homozygous for methionine at codon 129, indicating that homozygotes have a higher risk of developing sCJD compared to heterozygotes (Palmer *et al.*, 1991). So far, the methionine homozygosity at codon 129 seems to be the only risk factor for developing sCJD since it is fully documented and statistically significant. However, two recent case control studies suggest different risk factors. It has been found that sCJD is more common in patients who have undergone frequent surgery and in people with residence or employment on farms or in market gardens (Collins *et al.*, 1999). Furthermore, sCJD is described more often in families with members that have died of dementia due to other causes than sCJD (van Duijn *et al.*, 1998). None of these studies has been confirmed so far.

1.4.3 Familial Creutzfeldt-Jakob disease (fCJD)

The familial forms of CJD accounts for 5-15% of all CJD patients. They include point mutation in the *Prnp* gene and insertions or deletions in the octarepeat region of the gene (Fig.1) (Goldfarb *et al.*, 1994). Familial CJD with the codon 200 mutation (E K) is the most frequent and has been reported in geographical clusters. Libyan Jews in Israel (Goldfarb *et al.*, 1990a; Hsiao *et al.*, 1991), Spanish families in rural Chile (Goldfarb *et al.*, 1994), and families in central Slovakia were affected (Goldfarb *et al.*, 1990b). Furthermore, isolated familial cases have been reported in Canada, France, Japan, the United States and the United Kingdom (Goldfarb *et al.*, 1994).

Familial CJD with a codon 178 mutation (D N) combined with valine at the polymorphic codon 129 has been reported in families originating from England, Finland, France, Hungary, and the Netherlands (Goldfarb *et al*., 1992). Pedigree analyses of the Finnish family indicated that codon 178 mutation could have a disease penetration rate of 100% (Goldfarb *et al.*, 1994).

The clinical signs, duration and onset of the disease can vary, depending on the mutation type. Patients with codon 200 mutation closely resemble the phenotype seen in sCJD with a mean duration of 8 months and a mean onset at 55 years of age (Brown *et al.*, 1991b), whereas the codon 178 mutation shows a different phenotype with a longer duration of the disease (mean, 23 months and an earlier age of onset (mean, 46 years) (Brown *et al.*, 1992).

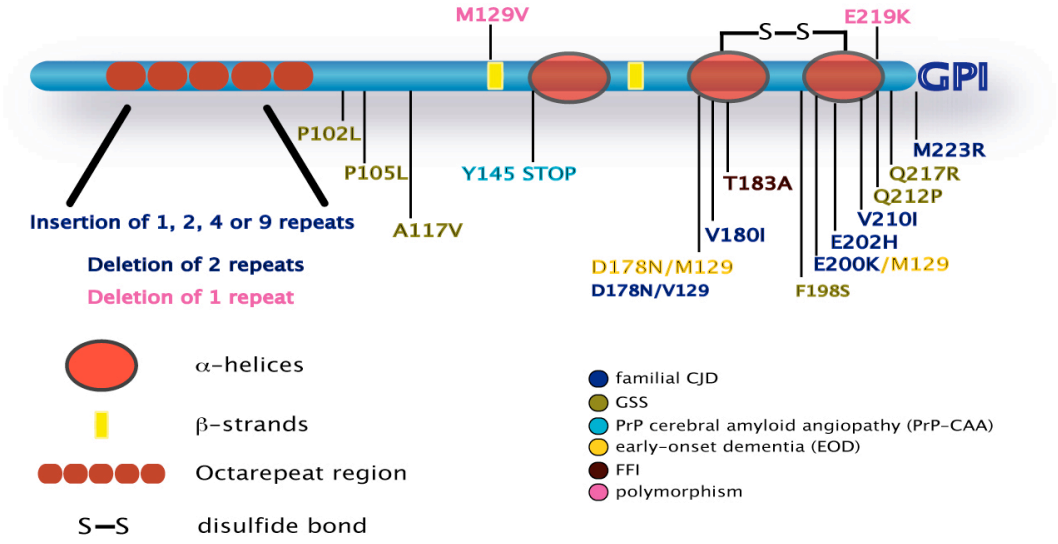


Fig. 1 Mutations and polymorphisms of the human prion protein that are associated with familial human prion diseases. Main structural elements of the prion protein are shown.

1.4.4 New Variant Creutzfeldt-Jakob disease (vCJD)

In 1990, the National CJD Surveillance Unit (NCJDSU) in the UK started its work. The aim was to identify and characterize sporadic CJD cases that did not match the usual disease characteristics. About 5 years later, in 1995 and early 1996, 10 cases of sCJD in remarkably young patients were reported to the NCJDSU (Bateman *et al.*, 1995; Britton *et al.*, 1995). The article 'A new variant of Creutzfeldt-Jakob disease in the UK' was published in April 1996 and drew a causal link between the epidemic of BSE in cattle in the UK and new variant CJD (vCJD) (Will *et al.*, 1996). The most prominent characteristics of this new variant of CJD are

the young age of the patients and the existence of 'floride plaques' in the brain of the patients (Will *et al.*, 1996). Recently, vCJD was described in a 74-year-old patient (Lorains *et al.*, 2001). This report, and a very recent report of BSE-infected mice with both sporadic and variant CJD-like symptoms (Asante *et al.*, 2002) tells us that also old patients and patients with symptoms similar to sporadic CJD can be infected with the BSE agent. Now, there is compelling evidence for a direct transmission of BSE from cattle to human beings:

- It was shown that brains of macaques, when inoculated with the BSE agent, show the same characteristic 'floride' plaques seen in vCJD patients (Lasmézas *et al.*, 1996).
- PrP^{Sc} in brains of mice, domestic cat and macaque that have been inoculated with PrP^{BSE} show the same glycosylation pattern as PrP^{Sc} from brain of vCJD patients (Collinge *et al.*, 1996).
- Transgenic mice, expressing human PrP only, can be infected with the BSE agent (Hill *et al.*, 1997).
- Mice expressing wild type PrP have been infected with the BSE agent and the vCJD agent. Both show identical lesion profiles and incubation time (Bruce *et al.*, 1997).
- Using a cell free conversion assay human PrP^{C} could be converted to the abnormal isoform by using PrP^{BSE} as a seed (Raymond *et al.*, 1997).
- Transgenic mice, expressing bovine PrP only, have been infected with the BSE- and the vCJD agent. Both show the same incubation time, PrP^{Sc}-isotype and neuropathology (Scott *et al.*, 1999).

The different studies show that it is most likely that BSE is transmissible to humans. A very recent study postulated the existence of two different disease patterns in transgenic mice challenged with BSE prions (Asante *et al.*, 2002). The transgenic mice do express human PrP with methionine at codon 129. After BSE infection some mice show type 4 PrP^{Sc}, which is similar to PrP^{Sc} seen in vCJD patients. Surprisingly, other mice show type 2 PrP^{Sc}, which is the most common strain in sporadic CJD patients. This data suggests that patients with a phenotype related to sporadic CJD might also have been infected with BSE prions. This finding might explain the recently increased number of sCJD cases in Switzerland and in the UK.

1.4.5 Iatrogenic Creutzfeldt-Jakob disease (iCJD)

Iatrogenic transmission of CJD was first suggested in 1974 in the recipient of a corneal transplant from a donor who died from CJD. Most cases resulted from exposure to infectious brain (dura mater grafts) and cadavric pituitary tissue derived hormones like gonadotropin and human growth hormone. In both forms the homozygosity for methionine at codon 129 is overrepresented and might have some influence on the incubation period of hormone treated patients, whereas no effect on the incubation period by the codon 129 polymorphism of graft recipients was observed. The proportion of patients acquiring CJD from growth hormone can vary from 0.3 to 4.4% in different countries, and acquisition from dura mater varies between 0.02 and 0.05% in Japan (where most cases occurred) (Brown *et al.*, 2000).

1.4.6 Fatal Familial Insomnia (FFI)

The term FFI was first used in 1986 by Lugaresi and co-workers to describe progressive insomnia and autonomic dysfunction, followed by dysathria, tremor, and myoclonus in a 52-year-old male in Italy. The patient's two sisters and many other relatives over three generations died of a similar disease (Lugaresi *et al.*, 1986). However, in 1939, cases with severe dementia were described and probably represent early cases of an FFI like disease (Stern, 1939). The onset of the disease is usually in the fifth decade and ranges from 35-61 years of age and the duration is 13 months in mean (Manetto *et al.*, 1992; Medori *et al.*, 1992). The predominant feature in most FFI patients is involvement of the thalamus associated with severe sleep disturbances, often with insomnia, and autonomic dysfunction (Manetto *et al.*, 1992). Although rare sporadic cases have been reported recently, FFI is a predominantly familial disease with a mutation in *Prnp* gene at codon 178 (D N) in combination with methionine at the polymorphic codon 129. The illness duration has been reported to be significantly shorter with methionine homozygosity (mean, 12 month) compared to patients with methionine/valine heterozygosity (mean, 21 month) (Gambetti and Lugaresi, 1998). Very recently, insomnia associated with thalamic involvement was reported in a case of fCJD, where the patient's *Prnp* gene showed the E200K mutation and homozygosity for methionine at codon 129 (Taratuto *et al.*, 2002).

1.4.7 Gerstmann-Sträussler-Scheinker Syndrome (GSS)

GSS, a familial disease with autosomal dominant inheritance, was first described in 1936 by Gerstmann, Sträussler and Scheinker (Gerstmann *et al.*, 1936). Since then the name GSS has been used to describe a group of neurodegenerative disorders with a familial origin closely related to CJD. GSS is primarily associated with mutations at codon 102 and less frequently with mutations at other codons of the prion protein (Fig.1) (Cervenakova *et al.*, 1999; Doh-ura *et al.*, 1989; Gajdusek, 1996). The most frequent mutation at codon 102 (P L) is associated with a comparably early onset of disease (mean, 48 years), and a prolonged duration of illness (mean, 5 years) (Brown *et al.*, 1991a). Slowly progressive gait abnormalities and ataxia are the common clinical features of this mutation. In contrast to this so-called 'ataxic GSS', the mutation at codon 117 (A V) is associated with a 'dementing' phenotype (Hsiao and Prusiner, 1990).

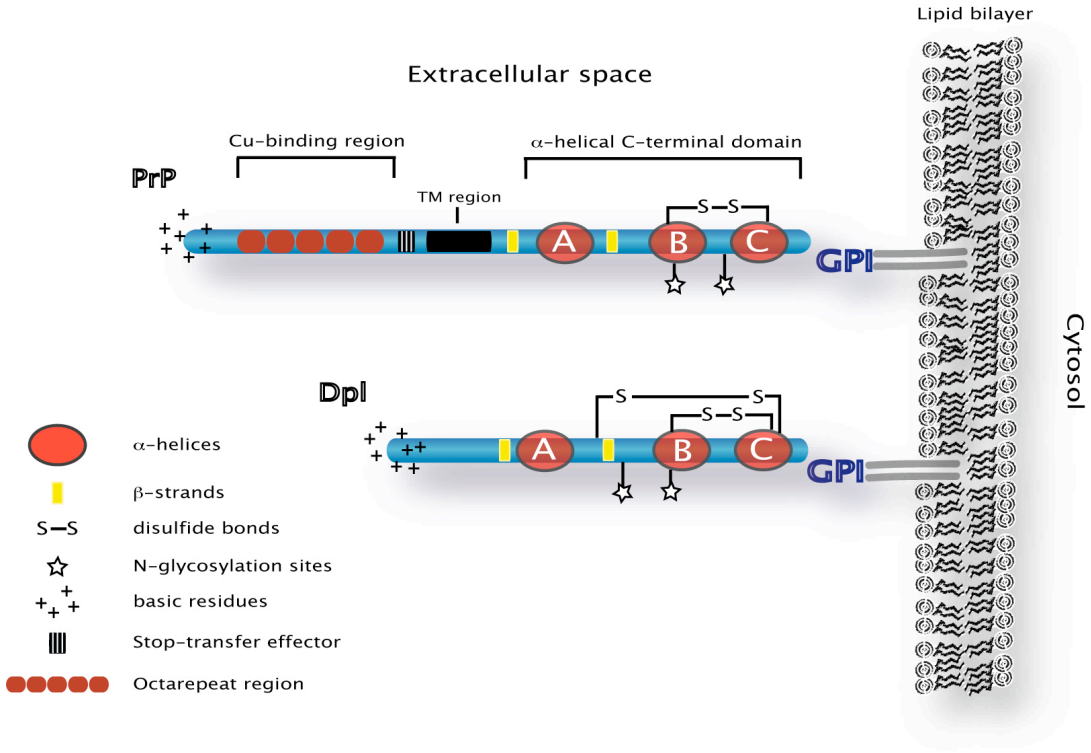


Fig. 2 Structural elements of the prion protein (PrP) and its homolog Doppel (Dpl). Both Proteins are anchored in the plasma membrane with a glycosylphosphatidylinositol mojety, orientated towards the extracellular space. PrP and Doppel have similar C-terminal domains, whereas Dpl lacks a Cu-binding region. The stop-transfer effector (STE) is involved in the formation of the transmembrane form of PrP (^{Ctm}PrP).

1.5 Structure, function, and trafficking of PrP

Mammalian prion proteins are transported via the endoplasmatic reticulum and secretory vesicles to the cell surface, where they are anchored by glycosylphosphatidylinositol (GPI) membrane anchor (Fig.2) (Caughey and Raymond, 1991; Stahl *et al.*, 1987). There, the prion protein resides for about 60 minutes before it becomes internalized via clathrin-coated pits or caveolae-like domains (CLDs) (Shyng *et al.*, 1994; Vey *et al.*, 1996). All mammalian prion proteins are encoded by a single exon as a polypeptide chain of about 250 to 260 amino acid residues, depending on the species (Oesch *et al.*, 1985; Schätzl *et al.*, 1995; Wopfner *et al.*, 1999). After the cleavage of an N-terminal signal peptide of 22 residues and a C-terminal signal peptide of about 23 residues the mature prion protein consists of polypeptide chain of about 210 residues in length (Basler *et al.*, 1986; Bazan *et al.*, 1987). PrP^{C} has a single disulfide bridge between cystein 179 and cystein 214 (human PrP) and two N-glycosylation sites (Turk *et al.*, 1988). It was shown that PrP^{C} contains various glycosylation variants with more than 52 different bi-, tri-, and tetra-antennary N-linked oligosaccharides (Rudd *et al.*, 1999). In addition to a GPI-anchored form of the prion protein, the existence of two other membrane-bound forms was observed: a C-terminal transmembrane form ^{Ctm}PrP, a N-terminal transmembrane form ^{Ntm}PrP (Hegde *et al.*, 1998). ^{Ctm}PrP and ^{Ntm}PrP span the membrane once via a conserved hydrophobic domain with either the C-or the N-terminus translocated to the ER lumen (Fig.2). One of these forms, ^{Ctm}PrP, triggers spontaneous neurodegeneration when overexpressed (Hegde *et al.*, 1998).

So far, all high resolution structural studies have used recombinant PrP^{C}, expressed in *E.coli*. However, with optical spectroscopy of natural PrP^{C} from hamster brain it was possible to elucidate the secondary structure of PrP^{C}. Circular dichroism and infrared spectroscopy resulted in spectra typical for an α-helical protein with predominantly α-helical (42%) and minor β-sheet (3%) content (Caughey *et al.*, 1991; Pan *et al.*, 1993; Safar *et al.*, 1993). In the same studies the secondary structure of PrP^{Sc} derived from brain was shown to have a high β-sheet content (>40%) and less α-helical structures ($\leq$20%). These findings are in close agreement with all high resolution NMR structures that have been recorded so far. The first structure which has been published was that of recombinant mouse PrP^{C} from residue 121-

231 in 1996 (Riek *et al.*, 1996). This was followed by the NMR structure of mouse PrP^C (aa23-231) (Riek *et al.*, 1997), hamster PrP^C (aa90-231) (Liu *et al.*, 1999), human PrP^C (aa-23-230) (Zahn *et al.*, 2000) and bovine PrP^C (aa25-242) (Lopez Garcia *et al.*, 2000). All four presently available NMR structures of PrP^C have the same structural architecture: a flexible, apparently unstructured N-terminal tail and a well-ordered C-terminal domain consisting of three α-helices and two short, antiparallel β-strands. The flexible tail represents an unusual feature of proteins compared with the presently available protein structure database.

In 2001, a crystal structure of a recombinant PrP^C dimer was published (Knaus *et al.*, 2001). The dimerization involves a three-dimensional swapping of the C-terminal helix 3 and rearrangement of the disulfide bond. The authors suggest that the observed dimer might represent a first step in oligomerization and subsequent amyloidosis in prion diseases. However, it is not clear if the dimeric structure is present under physiological conditions because of harsh crystallization conditions used in this study. Very recently, a crystal structure of a copper binding PrP fragment in association with copper was recorded (Burns *et al.*, 2002).

1.5.1 The conversion of PrP^C to PrP^{Sc}

No differences in the primary structure of PrP^C and PrP^{Sc} have been detected, suggesting that they might differ in their conformation (Stahl *et al.*, 1993). However, the tertiary structure of PrP^{Sc} remains unclear. The protein-only hypothesis tells us that PrP^{Sc} might be the only constituent of the infectious agent (Prusiner, 1998). Within this hypothesis two models have been proposed to explain the conversion of the host encoded cellular prion protein to the abnormal form PrP^{Sc}. The 'refolding model' postulates that PrP^C unfolds partly and then refolds under influence of a PrP^{Sc} molecule. Normal and misfolded state are separated by an activation energy barrier (Prusiner, 1991). The 'nucleation model' proposes that PrP^C is in equilibrium with PrP^{Sc}, that the equilibrium is largely in favor of PrP^C and that PrP^{Sc} is only stable when forming multimers (Jarrett and Lansbury, 1993). Conversion *in vitro* from PrP^C to a PrP^{Sc}-like molecule was first demonstrated in 1994 (Kocisko *et al.*, 1994). Since then, this *in vitro* model has been used to elucidate species barrier and strain-specificities of PrP^{Sc} (Bessen

et al., 1995; Raymond *et al.*, 1997). However, due to a less than stoichiometric yield with respect to PrP^{Sc} used as a seed, it was not possible to demonstrate an increase in infectivity. Recombinant PrP^{C} has been converted to a β-sheet-rich, partially protease resistant state by using physico-chemical procedures (Jackson *et al.*, 1999; Lu and Chang, 2001). So far, there have been no reports that such material gives rise to transmissible spongiform encephalopathies (Shaked *et al.*, 1999).

1.5.2 The function of PrP^{C}

So far, several different approaches to clarify the cellular function of PrP^{C} have been made resulting in different cellular and biochemical properties of the prion protein.

A common strategy to determine protein function is to ablate the gene of interest and examine homozygous null mice for novel phenotypes. In the case of PrP knock-out mice ($Prnp^{0/0}$) the loss of PrP^{C} is not associated with any phenotype (Lledo *et al.*, 1996; Manson *et al.*, 1994), despite a resistance towards scrapie infection (Bueler *et al*., 1993). However, in other reports $Prnp^{0/0}$ mice showed a loss of Purkinje cells, late-onset ataxia, alterations in synaptic processes and altered circadian activity (Sakaguchi *et al.*, 1996) (Collinge *et al.*, 1994; Tobler *et al.*, 1996). The loss of Purkinje cells was later assigned to the upregulation of the prion-like protein Doppel (Dpl) in this particular strain of knock-out mice, rather than to the deletion of PrP^{C} (Moore *et al.*, 1999). Like PrP, Dpl is an N-glycosylated and GPI-anchored membrane-bound protein, but lacks the octarepeat region and is predominantly expressed in testis (Silverman *et al.*, 2000). The level of Dpl in mouse brain inversely correlates with the onset of ataxia and the loss of Purkinje cells (Rossi *et al.*, 2001). It was hypothesized that the lack of PrP^{C} in $PrP^{0/0}$ mice might be counterbalanced by an altered expression of other genes during embryogenesis and therefore no phenotype is observed. Two different approaches were used to test this hypothesis:

- PrP^{C}-expression was established in a $PrP^{0/0}$ genetic background controlled by a regulative transcriptional *trans*-activator (tTA/tetO-*Prnp*). Administration of doxycycline to adult tTA/tetO-*Prnp* transgenic mice resulted in reduction of PrP^{C}

levels to 10% compared to wild type mice but had no effect on neuronal viability (Tremblay *et al.*, 1998).

- The coding region of PrP was deleted using Cre recombinase at an age of nine weeks. This post-natal knockout did not induce neurodegeneration or pathological changes for up to 15 months post-knockout. However, afterhyperpolarization potentials (AHPs) in hippocampal CA1 cells were significally reduced, suggesting a role for PrP^{C} in the modulation of neuronal excitability (Mallucci *et al.*, 2002).

Copper binding seems to be one of the most prominent features of PrP. It was shown in several publications

- that PrP binds copper *in vitro* and *in vivo*, and
- that the binding of copper influences internalization of PrP (Brown *et al.*, 1997; Jackson *et al.*, 2001; Lee *et al.*, 2001; Pauly and Harris, 1998)

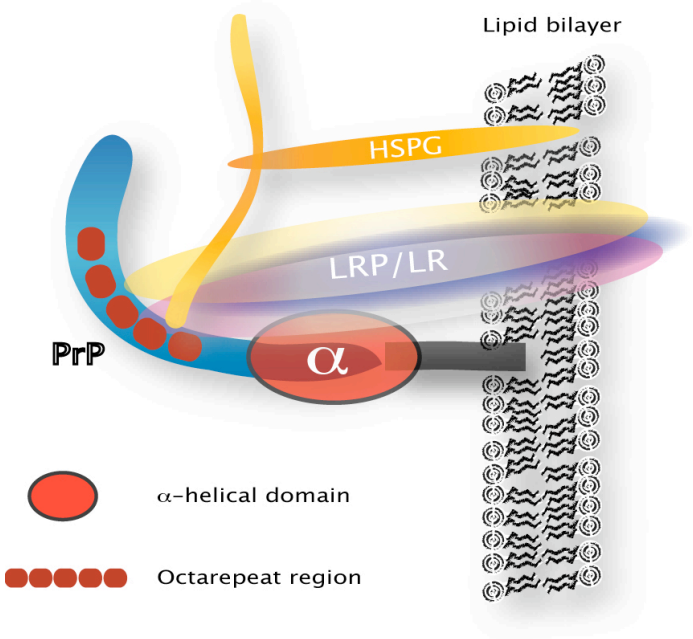


Fig. 3 Interaction of PrP and LRP/LR involving HSPG's. LRP/LR binds to PrP via the C-terminal region (aa 144-179) and a second, HSPG dependent binding domain (aa 53-93) located within the octarepeat region of PrP.

Furthermore, binding of PrP^C to at least 16 proteins and other macromolecules has been proposed (for review (Gauczynski *et al.*, 2001a)). One of these is the 37-kDa/67-kDa laminin receptor (LRP/LR), that has been identified employing a yeast-two hybrid screen (Rieger *et al.*, 1997). Via binding to a transmembrane receptor, such as the 37-kDa/67-kDa laminin receptor, PrP^C is able to trigger its own internalization (Gauczynski *et al.*, 2001b) and may induce other signalling events like the activation of tyrosine kinase Fyn (Mouillet-Richard, 2000).

Heparan sulfate proteoglycans (HSPGs) have been characterized as a major binding partner of PrP^C (Gabizon *et al.*, 1993; Shyng *et al.*, 1995a) (Hundt *et al.*, 2001). HSPGs are thought to form a complex with PrP in association with the 37-kDa/67-kDa laminin receptor (Fig.3) (Hundt *et al.*, 2001) and act as co-factors/co-receptors for PrP^C.

2 Role of the 37-kDa/67-kDa laminin receptor (LRP/LR) in prion diseases

In 1997, the 37-kDa/67-kDa laminin receptor (Fig.4) was identified as an interactor of the prion protein in eukaryotic cells (Rieger *et al.*, 1997). It was also shown that the LRP/LR protein level was elevated in organs of TSE-infected animals and scrapie-infected neuroblastoma cells, and that elevated LRP level correlated with PrP^{Sc}-propagation in different tissues (Rieger *et al.*, 1997). In further studies the direct interaction of PrP and LRP/LR *in vitro* and binding of recombinant PrP to LRP/LR on the cell surface was shown (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). Two distinct LRP/LR-binding domains on PrP were identified using yeast two-hybrid analysis and cell binding studies with recombinant PrP-peptides (Fig.3):

- PrP_{LRP}bd1 aa 144-179 (C-terminal domain)
- PrP_{LRP}bd2 aa 53-93 (octarepeat region)

Binding to the second binding domain has been shown to be mediated by heperan sulfate proteoglycans (Hundt *et al.*, 2001). Employing mutant CHO cells that lack HSPGs and a PrP-peptide comprising the octarepeat region of PrP it was shown that this peptide is not able to

bind to HSPG-deficient CHO cells (Hundt *et al.*, 2001). However, externally added HSPGs restored the binding of the peptide to these cells, demonstrating that HSPGs are needed for the binding of the PrP-peptide (Hundt *et al.*, 2001).

Furthermore, it has been shown that the presence of LRP/LR on the cell surface of mammalian cells is a prerequisite for binding and internalization of externally added PrP (for review see (Leucht and Weiss, 2002)). Blockage of LRP/LR by pre-incubation with LRP/LR specific antibodies resulted in an inhibited PrP-binding to the cells (Gauczynski *et al.*, 2001b). In addition, it was shown that externally added PrP is internalized by an LRP/LR-dependent mechanism (Gauczynski *et al.*, 2001b). By decreasing the incubation temperature from 37°C to 4°C, internalization of PrP was totally inhibited, showing that the PrP internalization process is an active, receptor mediated process (Gauczynski *et al.*, 2001b). At 37°C, 25-50% of the bound PrP was internalized in an LRP/LR dependent manner (Gauczynski *et al.*, 2001b).

The 37-kDa/67-kDa laminin receptor has two major isoforms, the mature 67kDa laminin receptor and the 37kDa laminin receptor precursor. Both forms have been shown to locate in

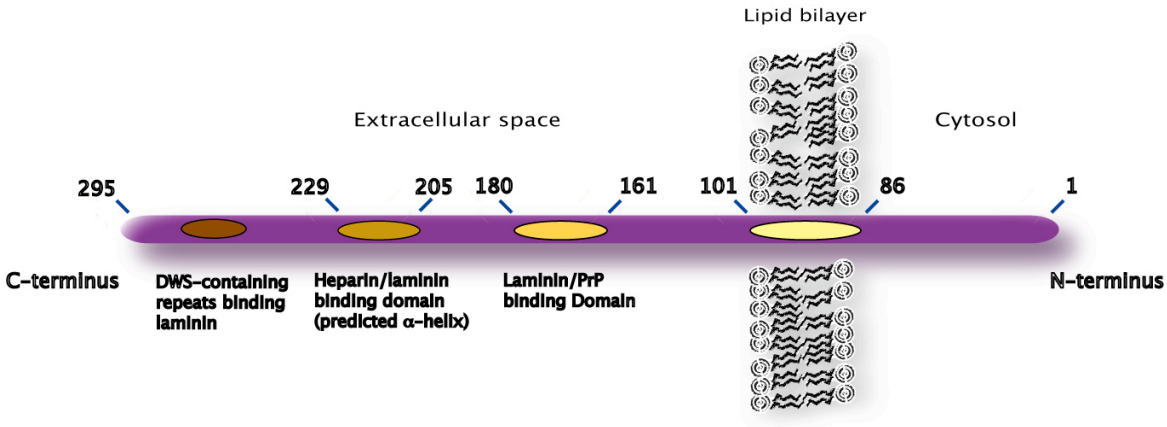


Fig. 4 Functional domains of LRP/LR. LRP/LR spans the plasma membrane once with its C-terminus directed towards the extracellular space (Castronovo *et al.*, 1991b). PrP (Rieger *et al.*, 1997), laminin (Castronovo *et al.*, 1991b)and heparin (Kazmin *et al.*, 2000) binding sites have been characterized.

the plasma membrane of neuronal cells (Gauczynski *et al.*, 2001b). However, four PrP-binding isoforms were identified in mouse brain, including a 60 kDa and a 220 kDa form (Simoneau *et al.*, in press).

In parallel to the discovery of LRP/LR as a PrP receptor a second protein was found to interact with the prion protein on the cell surface of mammalian cells (Martins *et al.*, 1997). This protein has an apparent molecular weight of 66 kDa and has been identified using complementary hydropathy. There has been plausible theorizing about the 66 kDa protein being the same protein as LRP/LR and indeed, both proteins are found in brains of mice, share the same electrophoretic pattern with a doublet band at 60/67 kDa, and display the same binding behaviour when used in overlay assays together with PrP (Simoneau *et al.*, in press). In contrast to our findings the 66 kDa protein was recently identified as the murine stress-inducible protein 1 (STI-1) (Zanata *et al.*, 2002). STI1 is thought to interact with PrP^{C} on the cell surface inducing neuroprotective signals that rescue cells from apoptosis (Zanata *et al.*, 2002).

In addition to the previous mentioned influences of LRP/LR on the metabolism of the cellular prion protein, PrP^{C}, the effect of LRP/LR blockage on the abnormal prion protein, PrP^{Sc}, has been investigated (Leucht et al., in press). Scrapie infected neuroblastoma cells were used as a model system to elucidate the effect of LRP/LR ablation on the abnormal prion protein in these cells. The knock-down of LRP/LR expression by transfection of an expression plasmid carrying an antisense LRP RNA cassette and transfection of siRNAs specific for the LRP cDNA resulted in a transient reduction of PrP^{C} and in an long lasting decrease of PrP^{Sc} (Leucht et al., in press). The same effect was seen by blocking LRP/LR on the cell surface with specific antibodies. Most likely these effects are due to inhibited internalization of PrP^{C} which therefore might not be convertible to PrP^{Sc}, and to an inhibited direct binding of LRP/LR to PrP^{C} or PrP^{Sc}. However, little is known about the conversion process and it has to be investigated wether LRP/LR might be involved in the conversion process.

In summary, LRP/LR plays a crucial role in the metabolism of the normal prion protein and is required for PrP^{Sc} propagation witin the life cycle of prions.

CHAPTER II

DER PRION-PROTEIN-REZEPTOR

published as:

Christoph Leucht and Stefan Weiss

Der Prion-Protein-Rezeptor.(2002) *Nova Acta Leopoldina*, BSE-Wahnsinn und Wirklichkeit, NF *87*, Nr. 327, 39-54

Zusammenfassung

Kürzlich konnten wir mit Hilfe eines *yeast two-hybrid screens* ein Protein identifizieren, daß spezifisch mit dem Prion-Protein (PrP) interagiert. Es handelt sich hierbei um das 37 kDa Laminin-Rezeptor Vorläuferprotein (37 kDa LRP) (Rieger *et al.*, 1997). Angetrieben durch diese Entdeckung, konnten wir durch mehrere Versuche die Bedeutung des 37 kDa LRP im Lebenzyklus von Prionen erarbeiten (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001; Rieger *et al.*, 1997). Die Interaktion zwischen 37 kDa LRP und PrP wurde durch Koinfektions- und Kotransfektionsstudien in Insekten- und Säugerzellen bestätigt (Rieger *et al.*, 1997). Weiterhin konnte eine Rezeptor/Ligand Bindungskonstante ermittelt werden und eine *in vitro* Interaktion zwischen rekombinantem PrP und LRP gezeigt werden (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). Es ist wahrscheinlich, daß LRP auch eine Rolle in der Pathogenese von Prionenerkrankungen spielt, denn Organe von an Prionenerkrankungen leidenden Tieren zeigen einen erhöhten Proteinspiegel des 37 kDa LRP und auch bei mit Prionen infizierten Zellen konnte auf Proteinebene ein erhöhter LRP Spiegel gezeigt werden (Rieger *et al.*, 1997). Bei Experimenten zur Internalisierung von rekombinantem PrP an Zellen, konnte gezeigt werden, daß dieser Vorgang abhängig vom 37 kDa/67 kDa Laminin-Rezeptor ist (Gauczynski *et al.*, 2001b). Umfangreiche *mapping* Experimente, mit Hilfe des *yeast two-hybrid* Systems und Zellbindungsstudien mit unterschiedlichen PrP-Peptiden zeigten, daß zwei Regionen im Prion-Protein existieren, an die LRP bindet. Eine Region (PrPLRPbd1), Aminosäure 144 bis 179, ist direkt, während die Bindung an die zweite Bindedomäne (PrPLRPbd2), die sich von Aminosäure 53 bis 93 erstreckt, durch Heperansulfat Proteoglykan (HSPG) Moleküle vermittelt wird (Hundt *et al.*, 2001), welche als Korezeptoren/Kofaktoren für Prion-Proteine fungieren.

Abstract

Recently, we identified the 37 kDa laminin receptor precursor (LRP) as an interactor for the prion protein (PrP), using a yeast two-hybrid screen (Rieger *et al.*, 1997). Employing several experimental approaches, we showed that the 37 kDa LRP plays an important role within the life cycle of prions. We could confirm the interaction of both proteins by cotransfection

studies in insect and COS-7 cells (Rieger *et al.*, 1997). Futhermore a binding constant was determined and an in vitro interaction of both proteins could be demonstrated (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). It is likely that LRP plays a role in the pathogenesis of prion diseases, since we observed an increased level of LRP in several organs from rodents, suffering from prion diseases, and in scrapie infected neuroblastoma cells (Rieger *et al.*, 1997). Binding and internalization experiments of cellular PrP showed, that this process is strictly dependent on the presence of the 37 kDa/67 kDa laminin receptor on the cell surface of mammalian cells (Gauczynski *et al.*, 2001b). Mapping studies, with the yeast two-hybrid system and cell binding studies indicate that there are two distinct regions on PrP which interact with LRP (Hundt *et al.*, 2001). One region (PrPLRPbd1) streching from amino acid 144 to 179 is direct, whereas the other region (PrPLRPbd2), which spans from amino acid 53 to 93, encompassing the octarepeat region, is dependent on heperan sulfate proteoglycans (HSPG's) (Hundt *et al.*, 2001).

1 Einführung

Übertragbare spongiforme Enzephalopathien sind neurodegenerative Erkrankungen, die durch Prionen (***pro****teinaceous* ***in****fectious particles*) ausgelöst werden. Dazu gehören die Creutzfeldt-Jakob Krankheit (CJD), Fatale Familiäre Insomie (FFI), Gerstman-Sträussler-Scheinker Syndrom (GSS), Kuru beim Menschen, bovine spongiforme Enzephalopathie (BSE) beim Rind und die Traberkrankheit (*scrapie*) beim Schaf (Lasmézas and Weiss, 2000; Weissmann and Aguzzi, 1997). Prionen sind in jedem Fall innerhalb einer Spezies übertragbar und bestehen wahrscheinlich nur aus dem abnormalen Prion-Protein (PrP^{Sc}) als infektiöse Komponente und nicht aus Nukleinsäuren, deshalb handelt es sich nicht um eine Viruserkrankung, sondern um eine vollkommen neue infektiöse Krankheit, die Prionen-Erkrankung (Prusiner, 1982), bei der das normale Prion-Protein PrP^{C} und damit auch die abnormale, krankheitsauslösende Form, PrP^{Sc}, von einem wirtseigenen Gen abgelesen wird (Prusiner, 1998). PrP^{Sc} und seine ca. 142 AS lange Proteinase K resistente Form, PrP 27-30, kommen im Gehirn von erkrankten Individuen vor, wo sie amyloide Ablagerungen bilden

(McKinley *et al.*, 1991). PrP^{C} und PrP^{Sc} unterscheiden sich nicht in ihrer Aminosäurezusammensetzung, sie bilden aber eine unterschiedlich gefaltete dreidimensionale Struktur (Konformation) aus. PrP^{C} weist einen hohen Anteil an α-Helices auf (42 %) und besitzt wenig β-Faltblatt Struktur (3 %), wogegen PrP^{Sc} weniger α-Helices (30 %), aber mehr β-Faltblatt Strukturen (45%) aufweist (Caughey *et al.*, 1991; Pan *et al.*, 1993).

1.1 Die BSE Krise und ihre Folgen

1986 wurde der erste Fall von Boviner Spongiformer Enzephalopathie (BSE) in England histopathologisch bestätigt. In den folgenden Jahren sind über 180.000 BSE Fälle in England aufgetreten. Bei der Frage um die Herkunft von BSE werden zwei Hypothesen diskutiert. Die S*heep-origin*-Hypothese geht davon aus, daß BSE durch die Interspezies-Übertragung von *scrapie* Prionen aus Schafen auf Rinder ausgelöst wurde (Wilesmith *et al.*, 1991). Die B*ovine origin*-Hypothese hingegen sieht den Ursprung von BSE in der Übertragung einer spontan aufgetretenen BSE-Erkrankung beim Rind auf die gesamte Rinderpopulation in Großbritannien (Philips, 2000).

1996 ist im Zusammenhang mit der BSE-Krise eine neuartige Variante der Creutzfeldt-Jakob Krankheit (vCJD) aufgetreten (Will *et al.*, 1996). Das nahezu alleinige Auftreten dieser Krankheitsform in Großbritannien brachte einen ersten Hinweis auf eine Krankheitsübertragung von BSE infizierten Rindern auf Menschen. Bis jetzt (September 2002) sind über 130 Fälle von vCJD bekannt geworden, bei denen die Patienten außergewöhnlich jung waren, jedoch keine besonderen Essgewohnheiten hatten (Will *et al.*, 1999). Einen starken Hinweis auf die direkte Übertragung von BSE-Prionen auf den Menschen, brachten mehrere Versuche.

- Makkaken, die mit BSE Prionen inokuliert wurden, zeigten die gleichen floriden Plaques wie vCJD Patienten (Lasmézas *et al.*, 1996).
- Transgene Mäuse, die nur das humane Prion-Protein exprimieren, lassen sich mit BSE Prionen infizieren (Hill *et al.*, 1997).

- Wildtyp-Mäuse die mit vCJD Prionen oder BSE Prionen inokuliert wurden, zeigten vergleichbare Inkubationszeiten und Läsionsprofile (Bruce *et al.*, 1997).
- Bei *in vitro* Konversionsstudien konnte humanes PrP durch PrP^{BSE} in die Proteinase K resistente Form überführt werden (Raymond *et al.*, 1997).
- Mäuse, die ausschließlich das bovine Prion-Protein exprimieren, wurden mit BSE Prionen, sowie mit vCJD Prionen inokuliert und zeigten in beiden Fällen vergleichbare Inkubationszeiten, neuropathologische Merkmale und PrP^{Sc} Ablagerungen (Scott *et al.*, 1999).

Zusammenfassend kann nunmehr davon ausgegangen werden, daß die neue Variante der Creutzfeldt-Jakob Krankheit durch mit BSE Prionen kontaminierte Lebensmittel oder andere Produkte, die Rinderbestandteile enthalten, ausgelöst wurde.

1.2 Die Rolle eines zellulären Prion-Protein-Rezeptors

Die *Protein-only*-Hypothese sagt aus, daß das Zelloberflächenprotein PrP^{C} in seine krankheitsauslösende Isoform PrP^{Sc} überführt wird, ein Prozess bei dem die Konformation des Prion-Proteins einer dramatischen Umwandlung unterworfen ist (Prusiner *et al.*, 1998). Bei dieser Umwandlung erwirbt das Protein zusätzliche β-Faltblatt Regionen, welches zu einer partiellen Proteaseresistenz des Proteins führt. Von großem Interesse ist nun die Aufklärung des Lebenszyklus von Prionen in der Zelle, um den Ort der Konversion näher zu bestimmen (Abb. 1). PrP^{C} wird im rauhen endoplasmatischen Retikulum (rER) synthetisiert und wird über den Golgi Apparat und sekretorische Vesikel an die Zelloberfläche gebracht. Dort ist es über einen Glykosylphosphatidylinositolanteil (GPI) an der Plasmamembran verankert (Stahl *et al.*, 1987). Nach einer gewissen Verweildauer an der Zellmembran, welche bei Neuroblastomzellen ungefähr 60 Minuten beträgt (Shyng *et al.*, 1994), wird es über *clathrin-coated pits* (Shyng *et al.*, 1994) oder *caveolae-like domains* (CLD's) (Vey *et al.*, 1996) aufgenommen. Die Endozytose könnte durch ein Transmembranprotein, wie den Laminin-Rezeptor, vermittelt werden, welcher das GPI-verankerte Prion-Protein mit Clathrin verbindet. Von Harris wurde die Existenz eines PrP-Rezeptors vorgeschlagen, der eine

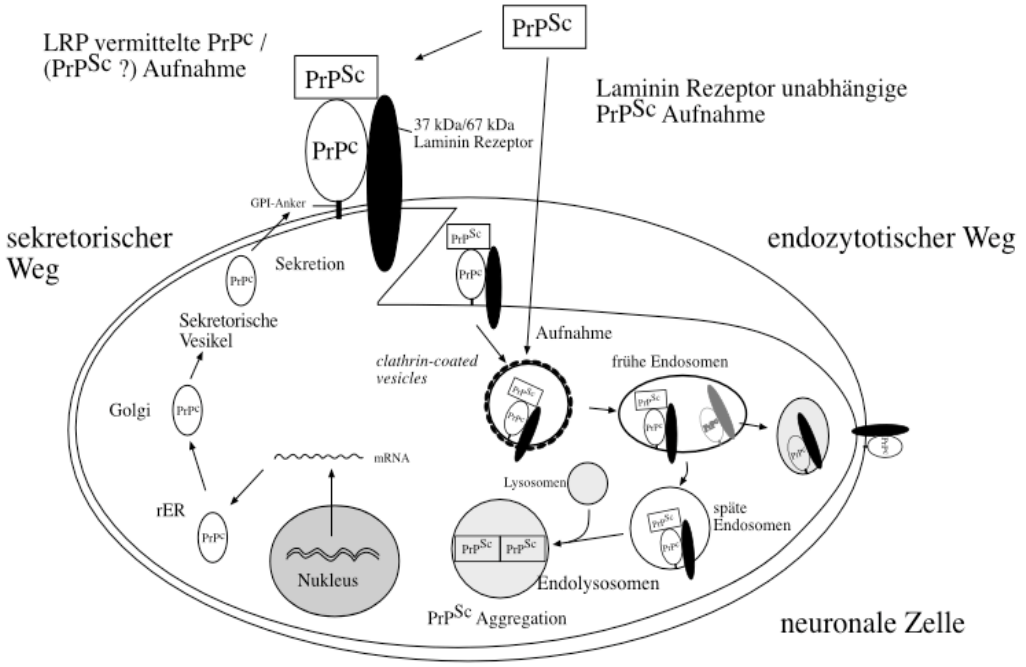


Abb.1 Modell für den Lebenszyklus von Prionen. PrP^C wird im rauhen Endoplasmatischen Retikulum (rER) synthetisiert und wird über den sekretorischen Weg an die Zelloberfläche gebracht, wo es über einen Glykosylphosphatidylinisitolanteil (GPI) verankert ist. Endozytose von PrP^C und möglicherweise PrP^{Sc} über *clathrin coated vesicles* wird vom 37 kDa/67 kDa Laminin-Rezeptor vermittelt. Die Aufnahme von PrP^{Sc} könnte auch unabhängig vom Laminin-Rezeptor stattfinden. Die Konversion des internalisierten PrP^C zu PrP^{Sc} findet vermutlich in Endosomen, Lysosomen oder Endolysosomen satt. PrP Replikation und Aggregation kann in neuronalen Zellen, sowie Zellen des lymphoretikulären Systems stattfinden. Alternativ dazu kann die Endozytose und Konversion von PrP^C zu PrP^{Sc} auch in *caveolae-like domains* (CLD's) stattfinden (modifiziert nach (Gauczynski *et al.*, 2001a; Gauczynski *et al.*, 2001b)).

C*oated-pit*-Lokalisationssequenz an seiner zytosolischen Domäne trägt und mit seiner extrazellulären Domäne an den N-terminalen Teil des Prion-Proteins binden kann (Harris, 1999; Harris *et al.*, 1996). Harris konnte desweiteren beobachten, daß N-terminal verkürzte Versionen des Prion-Proteins schlechter durch Endozytose in die Zelle aufgenommen werden und daß deren Konzentration in *coated-pits* weitaus geringer ist als die von Prion-Proteinen der vollen Länge (Harris, 1999; Shyng *et al.*, 1995b). Weiterhin konnte er beobachten, daß

Hühner Prion-Protein über Heperansulfat an die Zelloberfläche von Säugerzellen bindet (Shyng *et al.*, 1995a). Die Interaktion zwischen Heperansulfaten und dem Prion-Protein wurde bereits von zahlreichen Gruppen beschrieben (Brimacombe *et al.*, 1999; Caughey *et al.*, 1994; Chen *et al.*, 1995; Gabizon *et al.*, 1993). Kürzlich konnte gezeigt werden, daß Heparin die Bindung von Kupferionen an PrP kompetitieren kann (Brimacombe *et al.*, 1999), was darauf hinweist, daß Heparin an die Oktarepeatregion des Prion-Proteins bindet, von der man weis, daß sie Affinität für Kupferionen besitzt (Brown *et al.*, 1997; Jackson *et al.*, 2001). *In vivo* können Heperansulfat-Proteoglykane an das Prion-Protein binden. Heperansulfat Proteoglykane (HSPG's) bestehen aus einem Proteinanteil, dem C*ore*-Prtotein, an das über einen aus vier Zuckermolekülen bestehenden *Linker* Glykosaminoglykane (GAG's) angehängt sind. GAG's sind unverzweigte, negativ geladene Zuckerketten, die zum Beispiel aus Heperansulfaten bestehen können. Kürzlich konnten wir drei Heperansulfat-Bindedomänen im Prion-Protein identifizieren (Warner *et al.*, 2002). HSPG's spielen vermutlich eine wichtige Rolle im Lebenszyklus von Prionen. Der Prozess der Aufnahme von PrP^{Sc} in eine Zelle ist noch weitestgehend unklar. Das infektiöse Prion-Protein könnte

- über einen Rezeptor in die Zelle gelangen
- PrP^{C} vermittelt oder
- unspezifisch aufgenommen werden.

Die Konversion des Prion-Proteins in die abnormale Form könnte nach der Aufnahme in endozytischen Kompartimenten, wie Endosomen, Lysosomen oder Endolysosomen geschehen. Diese Konversion könnte von einem zellulären Protein, dem Protein X, beeinflußt werden (Telling *et al.*, 1995). Dabei könnte es sich um ein molekulares Chaperon, wie Hsp60 handeln (Edenhofer *et al.*, 1996). Auch das Phänomen der Speziesbarriere kann mit einem speziesspezifischem Molekül, wie Protein X, erklärt werden. Dabei könnte Protein X generell mit dem Prion-Protein assoziiert vorliegen und somit die Bindung und Konversion von PrP^{Sc} anderer Spezies beeinflussen. Ein weiterer Weg in die Zelle könnte über Caveolae führen, in denen sich GPI verankerte Proteine befinden können (Anderson, 1993). Der Umwandlungsprozess von PrP^{C} zu PrP^{Sc} könnte auch in *caveolae like domains* (CLD's) stattfinden, da beide Formen des Prion-Proteins in diesem Kompartiment nachgewiesen

wurden (Vey *et al.*, 1996). Um nun den komplexen Vorgang der Umwandlung des Prion-Proteins zu verstehen ist es notwendig dessen Interaktion mit möglichen Rezeptorproteinen, sowie die Bedeutung von PrP-ähnlichen Proteinen wie Doppel (Dpl), zu untersuchen.

Doppel wurde entdeckt, als man versuchte den Phänotyp einiger $PrP^{0/0}$-Mäuse zu erklären (Li *et al.*, 2000; Moore *et al.*, 1999). (Sakaguchi *et al.*, 1996) konnten erfolgreich $PrP^{0/0}$-Mäuse generieren, die im Unterschied zu anderen $PrP^{0/0}$-Mäusen (Bueler *et al.*, 1992; Lledo *et al.*, 1996; Manson *et al.*, 1994) einen zum Wildtyp unterschiedlichen Phänotyp zeigten. Die Mäuse zeigten Ataxien und einen Verlust an Purkinjezellen im Cerebellum. Als man der Sache auf den Grund ging, stellte man fest, daß das zu PrP ähnliche Protein Doppel verantwortlich für diese Veränderungen ist. Doppel wird bei Wildtyp-Mäusen nur im Testis exprimiert, doch durch Deletion einer 1 kb großen Region 5' des Exons 3 des PrP Gens *Prnp* wurde auch der 3' Spliceakzeptor deletiert und dies führte bei den genannten $PrP^{0/0}$ Mäusen zu einer Überexpression von Doppel im Gehirn (Li *et al.*, 2000; Moore *et al.*, 1999; Silverman *et al.*, 2000). Diese Überexpression ist verantwortlich für die beobachteten Symptome der *Knockout* Mäuse, wobei gezeigt werden konnte, daß die Menge von Doppel im Gehirn von $PrP^{0/0}$ Mäusen invers proportional zum Beginn der Kranheitssympotme ist und die Anwesenheit von nur einem *Prnp*-Allel die Krankheitssymptome verhindern kann (Rossi *et al.*, 2001). Hierbei handelt es sich um das erste PrP ähnliche Protein, das bei Säugern entdeckt wurde. Es besteht aus 179 Aminosäuren und besitzt ca. 25 % Homologie zu allen anderen bekannten Prion-Proteinen. Das Dpl Gen, *Prnd*, befindet sich 16 kb stromabwärts des PrP Gens *Prnp*. Es ist wie PrP selbst ein N-glykosyliertes und mit Glykosylphosphatidylinositol verankertes Protein, besitzt aber nicht die Prion-Protein-typische Oktarepeatregion (Silverman *et al.*, 2000). Wie kann nun die Überexpression von Doppel in Gehirn von $PrP^{0/0}$-Mäusen zu solchen Veränderungen führen? Durch Experimente mit $PrP^{0/0}$-Mäusen, die N-terminal verkürzte Versionen des Prion-Proteins exprimierten, konnte gezeigt werden, daß Mäuse, die PrP ohne die Regionen von Aminosäure 32-121 oder 32-134 exprimieren Symptome von Ataxie und Zelldegeneration im Gehirn entwickeln (Shmerling *et al.*, 1998). Kleinere Verkürzungen, dagegen, hatten keinen Effekt. Wie bei den Symptomen, die durch die Überexpression von Doppel hervogerufen wurden, konnte auch hier die Entwicklung von

Krankheitssymptomen durch die Expression von PrP verhindert werden. Da Doppel eine gewisse Homologie mit dem C-Terminus von PrP hat, kann man davon ausgehen, daß es sich bei den von Doppel ausgelösten Krankheitssymptomen und den Effekten der verkürzten Prion-Proteine um vergleichbare Phänomene handelt. An diesem Punkt kommt der PrP-Rezeptor ins Spiel. Man kann postulieren, daß der PrP-Rezeptor zwar an die verkürzten Prion-Proteine und Doppel binden kann, aber es in beiden Fällen nicht möglich ist eine anzunehmende Funktion auszuüben. Dies könnte dann zu dem pathologischen Phänotyp führen (zur weiteren Übersicht siehe (Gauczynski *et al.*, 2001a)).

Kürzlich wurde eine Aktivität des Prion-Protein bei der Signaltransduktion vorgeschlagen, bei der das Prion-Protein die Tyrosinkinase Fyn aktiviert (Mouillet-Richard, 2000). Da PrP^C auf der Zelloberfläche GPI verankert vorliegt und Fyn an der Innenseite der Plasmamembran sitzt, ist es vorstellbar, daß ein Transmembranprotein die Interaktion zwischen Fyn und PrP vermitteln könnte. Auch hier könnte die Rolle eines Prion-Protein-Rezeptors zu sehen sein.

1.3 Der 37 kDa/67 kDa Laminin-Rezeptor

Der 37 kDa *laminin receptor precursor* (37 kDa LRP, p40, LBP) ist das Vorläuferprotein des 67 kDa *high affinity* Laminin-Rezeptors (67 kDa LR) (Tab. 1)(Rao *et al.*, 1989; Yow *et al.*, 1988). Die 67 kDa Form wurde zunächst aus Tumorzellen isoliert (Lesot *et al.*, 1983; Malinoff and Wicha, 1983; Rao *et al.*, 1983), wo das Protein eine hohe Affinität zu Laminin aufweist. Laminin ist ein Glykoprotein der extrazellulären Matrix, wo es in Anhaftung, Bewegung, Differenzierung und Wachstum von Zellen involviert ist (Beck *et al.*, 1990). Beide Formen des Laminin-Rezeptors existieren nebeneinander in Säugerzellen, was durch immunologische Studien von Membranfraktionen gezeigt werden konnte (Gauczynski *et al.*, 2001b). Die 37 kDa Form kommt auch im Zytosol vor, wo sie mit Ribosomen assoziiert ist und Aufgaben bei der Proteintranslation übernehmen kann (Auth and Brawerman, 1992; Sato *et al.*, 1999). Es wurde auch die Existenz dieses Proteins im Kern diskutiert, wo es bei der Aufrechterhaltung von Strukturen involviert sein soll (Kinoshita *et al.*, 1998; Sato *et al.*, 1996). Alle Forschungsergebnisse zusammengenommen, handelt es sich um ein multifunktionelles Protein, welches ausgehend von dem Genprodukt p40 zwei

unterschiedliche Formen bilden kann, in verschiedenen Zellkompartimenten vorkommt und dort unterschiedliche Funktionen ausübt. Die Aminosäuresequenz des 37 kDa/67 kDa Laminin-Rezeptors ist hochkonserviert, mit einer hohen Homologie bei Säugern (Rao *et al.*, 1989). Durch eine Evolutionsanalyse der Aminosäuresequenz konnte gezeigt werden, daß die palindromische Sequenz LMWWML verantwortlich für die Fähigkeit ist, Laminin zu binden. Diese Sequenz liegt im PrP-bindenden Bereich des 37 kDa LRP (Ardini *et al.*, 1998; Hundt *et al.*, 2001; Rieger *et al.*, 1997). Es scheint, daß das ribosomale Protein p40, das zunächst nicht die Fähigkeit besaß Laminin zu binden (Auth and Brawerman, 1992), im Laufe der Evolution durch Aminosäureaustausch und Einführung von posttranslationalen Veränderungen zu einem lamininbindenden Zelloberflächenprotein evolvierte, welches auch Elastin (Hinek *et al.*, 1988; Salas *et al.*, 1992) und Kohlehydratketten (für eine Übersicht siehe (Ardini *et al.*, 1998; Mecham, 1991; Rieger *et al.*, 1999) binden kann.

Die Laminin-Rezeptor Familie ist in vielen eukaryotischen Zellen hochkonserviert (Keppel and Schaller, 1991; Wewer *et al.*, 1986) und kann auch in *Archaea* gefunden werden (Ouzonis *et al.*, 1995). Der 37 kDa LRP fungiert als Rezeptor für das Venezuelanische Equine Enzephalitis Virus auf Moskitozellen (Ludwig *et al.*, 1996), während die 67 kDa-Form offensichtlich als Rezeptor für das Sindbis Virus dienen kann (Wang *et al.*, 1992)

Tab. 1 Charakteristiken des 37kDa/67kDa Laminin-Rezeptors

Isolation	37 kDa LRP/p40 cDNA (Rao *et al.*, 1989; Yow *et al.*, 1988), 67 kDa Laminin Rezeptor, isoliert aus Tumoren (Lesot *et al.*, 1983; Malinoff and Wicha, 1983; Rao *et al.*, 1983)
Vorkommen des 37 kDa LRP/p40 Gens	*Saccharomyces cerevisiae* (Davis *et al.*, 1992), *Arabidopsis thaliana* (Garcia-Hernandez *et al.*, 1994), *Drosophila melanogaster* (Kazmin *et al.*, 2000; Melnick *et al.*, 1993), der Seeigel *Urechis caupo* (Rosenthal and Wordeman, 1995), *Chlorohydra veridissima* (Keppel and Schaller, 1991), *Candida albicans* (Lopez-Ribot *et al.*, 1994) und das Archaebakterium *Haloarcula marismortui* (Ouzonis *et al.*, 1995)
Subzelluläre Lokalisation des 37 kDa LRP	Auf der Zelloberfläche von Moskito Zellen (Ludwig *et al.*, 1996), *Candida albicans* (Lopez-Ribot *et al.*, 1994) und Säugerzellen wie (MDCK) (Salas *et al.*, 1992); im Zytoplasma auf 40S Ribosomen (Auth and Brawerman, 1992; Sato *et al.*, 1999); im Nukleus (Sato *et al.*, 1996)
Molekulargewicht	37.000 (Laminin Rezeptor Vorläuferprotein) 67.000 (gereifter Laminin Rezeptor)
Bindungspartner von - 37 kDa LRP	Laminin (Rieger *et al.*, 1997), PrP^C (Rieger *et al.*, 1997), das Venezuelanische Equine Enzephalitis Virus (VEE) (Ludwig *et al.*, 1996); Interaktion mit den Histonen H2A, H2B und H4 (Kinoshita *et al.*, 1998)
- 67 kDa LR	Laminin (Beck *et al.*, 1990), Elastin und Kohlehydrate (zur Übersicht siehe (Ardini *et al.*, 1998; Mecham, 1991; Rieger *et al.*, 1999)), der Sindbis Virus (Wang *et al.*, 1992)
Funktionelle Domänen	Transmembran Domäne; AS 86-101 (Castronovo *et al.*, 1991b), Laminin Bindedomäne (Castronovo *et al.*, 1991b), PrP^C Bindedomäne (Rieger *et al.*, 1997)

Welche Prozesse liegen der Umwandlung der 37 kDa Form in das 67 kDa Protein zugrunde? Beide Proteine bestehen aus der 37 kDa-Komponente, wobei mehrere Vorschläge gemacht wurden, die größere Masse des reifen Proteins zu erklären. Homodimerisierung des 37 kDa-Proteins, wie auch die Bindung einer anderen Komponente wurden diskutiert (Castronovo *et*

al., 1991a; Landowski *et al.*, 1995). Andere Studien jedoch schlagen ein durch Fettsäuren stabilisiertes Heterodimer vor (Buto *et al.*, 1998). Kürzlich wurde gezeigt, daß der 67 kDa-Laminin-Rezeptor auch auf aktivierten humanen T-Lymphozyten vorkommt und dort zusammen mit Integrinen eine starke Affinität zu Laminin aufweist (Canfield and Khakoo, 1999). Zusammenfassend kann jedoch gesagt werden, daß die Umwandlung von der 37 kDa- in die 67 kDa-Form des Laminin-Rezeptors ungeklärt ist.

Der 37 kDa/67 kDa Laminin-Rezeptor ist durch mehrere Gene im Genom von Säugern vertreten. Beim Menschen sind es 26, bei der Maus 6 Kopien (Fernandez *et al.*, 1991; Jackers *et al.*, 1996b). Das Gen besteht aus sieben Exons und sechs Introns, wobei es sich bei den meisten Genkopien wahrscheinlich um Pseudogene handelt (Jackers *et al.*, 1996a). Bei der Maus gibt Hinweise, das mindestens zwei der sechs Gene aktiv sind und sich auf Chromosom 9 bzw. 6 befinden (Douville and Carbonetto, 1992; Fernandez *et al.*, 1991). Interessanterweise wurde kürzlich gezeigt, daß sich auf Chromosom 9 möglicherweise Genloci befinden, welche die Inkubationszeit von Prionen-Erkrankungen bei Mäusen beeinflussen (Stephenson *et al.*, 2000).

Das Gen, daß für den 37 kDa LRP codiert ist in vielen verschieden Spezies identifiziert worden, wie *Saccharomyces cerevisiae* (Davis *et al.*, 1992), *Arabidopsis thaliana* (Garcia-Hernandez *et al.*, 1994), *Drosophila melanogaster* (Melnick *et al.*, 1993), dem Seeigel *Urechis caupo* (Rosenthal and Wordeman, 1995), *Chlorohydra veridissima* (Keppel and Schaller, 1991), *Candida albicans* (Lopez-Ribot *et al.*, 1994) und dem Archaebakterium *Haloarcula marismortui* (Ouzonis *et al.*, 1995).

2 Der 37 kDa/67 kDa Laminin-Rezeptor ist der zelluläre Rezeptor für das zelluläre Prion-Protein

Neuste Forschungsergebnisse zeigen, daß der 37 kDa/67 kDa Laminin-Rezeptor der Rezeptor für das Prion-Protein ist. Hierbei wurden wesentliche Fragen, welche im Zusammenhang mit der Interaktion beider Proteine stehen, beantwortet. Liegen beide Proteine im selben Zellkompartiment vor? Ist die Interaktion spezifisch? Hat die Interaktion Einfluß auf den zellulären Lebenszyklus des Prion-Proteins? Welche Regionen des Prion-Proteins vermitteln die Interaktion? Sind Kofaktoren involviert?

2.1 Die zelluläre Lokalisation des 37 kDa/67 kDa Laminin-Rezeptors

Um eine Funktion als zellulärer Rezeptor ausüben zu können, muß zumindest eine der beiden Formen auf der Zelloberfläche von relevanten Zellen vorkommen. Nach bisherigen Wissensstand, sind beide Formen auf der Oberfläche von verschiedenen Zelltypen nachzuweisen. Der 37 kDa LRP dient als Rezeptor für das Venezuelanische Equine Enzephalitis Virus auf Moskitozellen (Ludwig *et al.*, 1996), ist auf der Oberfläche von *Candida albicans* lokalisiert (Lopez-Ribot *et al.*, 1994) und wurde auf der Oberfläche von Madin-Darby *canine-kidney* (MDCK)-Zellen gefunden (Salas *et al.*, 1992). Kommt das 37 kDa Laminin-Rezeptor-Vorläuferprotein auch in anderen Zellkompartimenten vor (Nukleus, Zytosol), so scheint die Verbreitung der 67 kDa-Form auf die Zelloberfläche beschränkt zu sein. Die 67 kDa-Form stellt den Rezeptor für das Sindbis Virus auf Hamsterzellen dar (Wang *et al.*, 1992) und ist eine der Hauptkomponenten auf der Oberfläche von manchen Tumoren, wo er eine entscheidende Rolle bei der Metastasierung spielt (Castronovo, 1993).

Kürzlich konnten wir nachweisen, daß das Prion-Protein und der 37 kDa/67 kDa Laminin-Rezeptor auf der Oberfläche von N2a Zellen, sowie mit rekombinanter Semliki-Forest-Virus-(SFV)-RNA kotransfizierten *Baby Hamster Kidney* (BHK) Zellen, kolokalisieren (Gauczynski *et al.*, 2001b), eine Voraussetzung für eine *in vivo* Interaktion beider Proteine. FACS Analysen (*fluorescence-activated cell sorting*)mit LRP-spezifischen Antikörpern zeigten, daß der Laminin-Rezeptor auf der Oberfläche von nativen Zellen verschiedenen Typs

vorkommt. Da durch eine FACS Analyse keine Unterscheidung zwischen den beiden Formen gemacht werden konnte, stellten wir Membranfraktionen von N2a und BHK Zellen her. So konnten wir zeigen, daß sowohl die 37 kDa-Form, als auch die 67 kDa-Form in Membranen von N2a Zellen vorkommen, nicht aber in mit für LRP-kodierender, rekombinanter Semliki-Forest-Virus-(SFV)-RNA transfizierten BHK Zellen. Im letzteren Fall konnte im Zellysat und in der Membranfraktion nur die 37 kDa-Form nachgewiesen werden. Insgesamt zeigen unsere, sowie ältere Studien, daß der Laminin-Rezeptor durchaus an der Zelloberfläche vorkommt und somit eine Interaktion mit dem Prion-Protein möglich ist. Nichtdestoweniger ist eine Unterscheidung zwischen beiden Formen des Laminin-Rezeptors schwierig, deshalb müssen wir annehmen, daß beide Formen die Fähigkeit besitzen, mit dem Prion-Protein zu interagieren.

2.2 HSPG Moleküle als Kofaktoren/Korezeptoren des Prion-Proteins

Das Laminin-Rezeptor-Vorläuferprotein (37 kDa-LRP) wurde in einem *yeast two-hybrid screen* als Interaktor für das Prion-Protein (PrP^C) identifiziert (Rieger *et al.*, 1997) (Zur Übersicht siehe (Gauczynski *et al.*, 2001a; Rieger *et al.*, 1999)). In der selben Arbeit konnte auch eine Interaktion zwischen 37 kDa-LRP und PrP^C in Insekten- und COS-7 Zellen

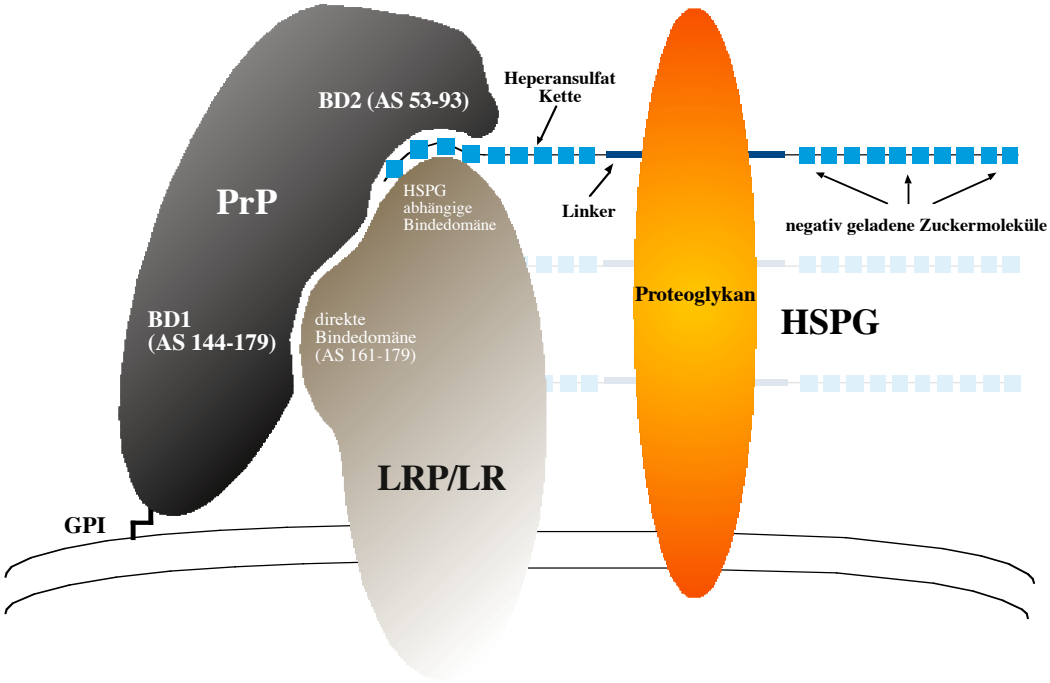


Abb. 2 Modell der Funktion des 37 kDa/67 kDa Laminin Rezeptors (LRP/LR) als Rezeptor für das Prion Protein. PrP bindet an LRP/LR über PrPLRPbd1 and PrPLRPbd2. PrPLRPbd2 (AS 53-93) ist abhängig von der Anwesenheit eines Heparansulfat (HS) Arms eines HSPG Moleküls. PrPLRPbd1 (AS 144-179) interagiert jedoch direkt mit LRP/LR (wie im *yeast two-hybrid* System gezeigt wurde). Die Anwesenheit von beiden Bindestellen, PrPLRPbd1 und PrPLRPbd2, könnte die Bindung des Prion Proteins beträchtlich stabilisieren. Direkte Bindung von LRP/LR an PrP findet über die direkte Bindestelle zwischen AS 161-179 auf LRP/LR statt. Die indirekte, HSPG abhängige Bindedomäne könnte sich zwischen AS 101 und 160 oder zwischen AS 180-285 (mutmaßlich AS 205 und 229 auf LRP (Kazmin *et al.*, 2000)) befinden. Die Assozation von LRP/LR mit HSPG's könnte die Beziehung zwischen 37 kDa LRP und 67 kDa LR erklären (modifiziert nach (Hundt *et al.*, 2001)).

nachgewiesen werden. Um weitere Indizien für die physiologische Relevanz dieser Interaktion zu sammeln, untersuchten wir die Bindung des reifen Prion-Proteins und verschiedener PrP-Peptide an lebende Zellen (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). Die Bindung eines Peptids an Säugerzellen ist dabei von Heparansulfat-Proteoglycanen (HSPG's) abhängig (Hundt *et al.*, 2001). Peptide, welche die Region des Prion-Proteins von Aminosäure 53-93 umfassen, binden nicht an HSPG-defiziente CHO Zellen, aber sehr wohl an Wildtyp-Zellen (Hundt *et al.*, 2001). Gleichzeitig wurde mit Versuchen im *Yeast-two-hybrid* System eine Region des Prion-Proteins als direkte Bindedomäne identifiziert (Hundt *et al.*, 2001). Hier wurden verkürzte Versionen des Prion-Proteins in der *Bait*-Position und das Laminin-Rezeptor-Vorläuferprotein in *Prey*-Position eingesetzt. Die Region von Aminosäure 144-179 des Prion-Proteins wurde als LRP bindende Region identifiziert. Diese Region unfasst die erste α-Helix und das zweite β-Faltblatt. Interessanterweise konnte kürzlich gezeigt werden, daß Anti-PrP-Antikörper, die gegen die Region der ersten α-Helix gerichtet sind PrP^{Sc} Vermehrung im Zellkuktursystem verhindern können (Peretz *et al.*, 2001). Weiterhin sind diese Antikörper fähig die Infektiösität in Prion infizierten Zellen aufzuheben. Peptide, die die Regionen von Aminosäure 144-179 des Prion-Proteins umfassen, konnten auch an Zellsystemen getestet werden, sie binden sowohl ohne, als auch in Anwesenheit von HSPG's an Zellen. Zusammenfassend konnten wir zwei Bindedomänen auf dem Prion-Protein identifizieren:

- PrPLRPbd1, von Aminosäure 144 bis 179 (direkte Bindedomäne)
- PrPLRPbd2, von Aminosäure 53 bis 93 (Heperansulfat Proteoglykan abhängige Bindedomäne) (Abb. 2).

Auf LRP konnten wir eine direkte Interaktionsdomäne mit PrP identifizieren (Rieger *et al.*, 1997), welche sich zwischen Aminosäure 157 und 180 befindet. Verkürzte LRP-Moleküle, LRP44-101 und LRP44-160, sowie LRP180-295 waren nicht in der Lage mit PrP der vollen Länge im *Yeast-two-hybrid*-System zu interagieren (Hundt *et al.*, 2001), was zeigt, daß die PrP Bindedomäne identisch mit der Laminin Bindedomäne ist. Auch eine direkte Interaktion zwischen PrPLRPbd1 und LRP161-179 konnte im *Yeast-two-hybrid*-System gezeigt werden (Hundt *et al.*, 2001), was beweist, daß sich die direkte Bindedomäne auf LRP von

Aminosäure 161 bis 179 erstreckt. Exprimiert man nun eine LRP Deletionsmutante, welche diese Binderegion nicht besitzt, in CHO-Zellen, so kann PrP^C nach wie vor an diese Zellen binden, was zeigt, daß eine zweite Bindedomäne für PrP auf LRP exisiert, welche sich zwischen Aminosäure 101-160 oder 181- 295 befindet (Hundt *et al.*, 2001). Kürzlich konnten wir zwei weitere Bindedomänen im zellulären Prion-Protein für Heperansulfate identifizieren (Warner *et al.*, 2002).

2.3 Die Bindung und Aufnahme von rekombinantem Prion-Protein durch Säugerzellen

Rekombinantes Prion-Protein kann von Säugerzellen gebunden und internalisiert werden (Gauczynski *et al.*, 2001b). Dabei untersuchten wir, ob dieser Vorgang von der Anwesenheit des Laminin-Rezeptors abhängig ist. Dazu wurden BHK Zellen mit rekombinanter für den Laminin-Rezeptor kodierender Semliki-Forest-Virus-(SFV)-RNA transfiziert. Die Zellen zeigten daraufhin eine Überexpression des humanen Laminin-Rezeptors, wobei nur die 37 kDa Form nachgewiesen werden konnte. Durch eine FACS Analyse konnte gezeigt werden, daß sich auch an der Zelloberfläche ein erhöhter Spiegel des Laminin-Rezeptors feststellen läßt. Dieser erhöhte Rezeptorspiegel auf der Plasmamembran erleichtert eine Bindung und Aufnahme des Prion-Proteins. Weiterhin konnte die Bindung und Aufnahme von PrP durch Vorabinkubation mit LRP spezifischen Antikörpern unterdrückt werden, was zeigt, daß die Bindung des Prion-Proteins von der Anwesenheit des Laminin-Rezeptors an der Zelloberfläche abhängig ist (Abb. 3). Wir stellten eine mengenabhängige Bindung des Prion-Proteins fest, welche in einer sigmoidalen Rezeptor/Ligand Bindungskurve resultierte. Ausgehend von dieser Kurve konnten wir eine Bindungskonstante (K_D) von 1×10^{-7} mol/L ermitteln (Gauczynski *et al.*, 2001b).

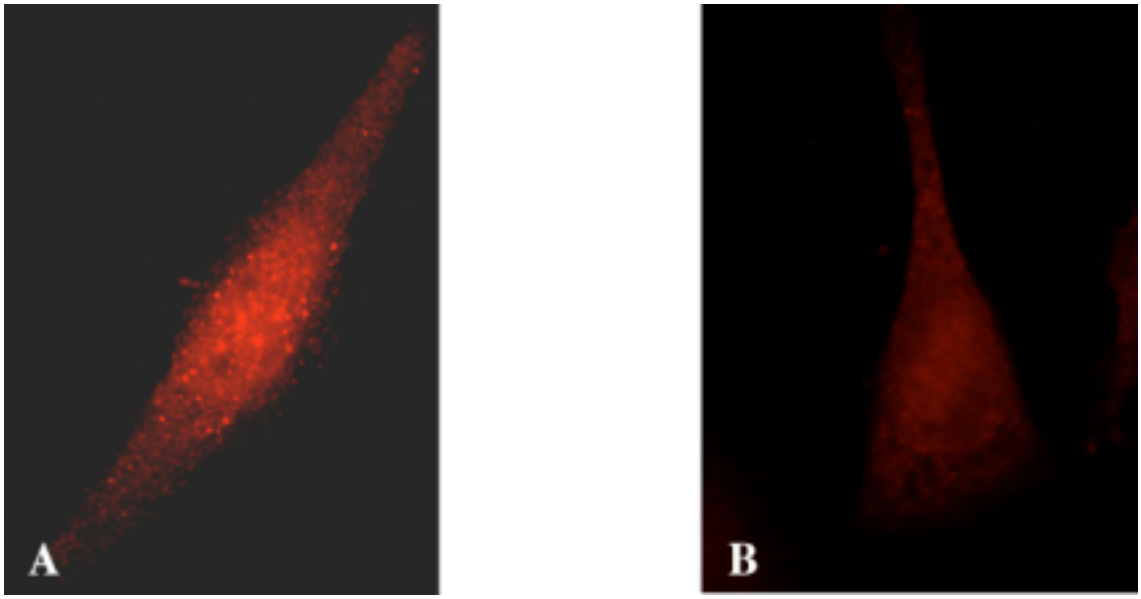


Abb.3 LRP/LR abhängige Bindung von rekombinantem PrP an N2a Zellen. **(A)** N2a Zellen wurden mit GST::huPrP23-230 (6 µg) inkubiert. Die Bindung des rekombinanten Proteins wurde über indirekte Immunfluoreszens sichtbar gemacht. Primärer Antikörper monoklonaler anti-PrP Antikörper 3F4, sekundärer Antikörper anti-Maus IgG Cy3; Zelloberflächenfärbung. **(B)** Vorabinkubation von N2a Zellen mit anti-LRP/LR spezifischem Antikörper (W3) verhindert die Bindung des rekombinantem prion proteins.

Doch spielt auch membranständiges Prion-Protein bei der Aufnahme von exogenem PrP eine Rolle? Wir isolierten primäre Neuronen aus $PrP^{+/+}$-, sowie $PrP^{0/0}$-Mäusen, um deren Aufnahme von exogenem, rekombinantem PrP zu Vergleichen. Es stellte sich heraus, daß die Aufnahme von PrP von diesen Neuronen wiederrum LRP-abhängig war und daß es keinen Unterschied in der Effizienz der Aufnahme gab (Gauczynski *et al.*, 2001b). Endogenes PrP dient also nicht als Korezeptor für die Aufnahme von exogenem PrP. Diese Versuche unterstreichen die wichtige Rolle des Laminin-Rezeptors als Rezeptor für das zelluläre Prion-Protein.

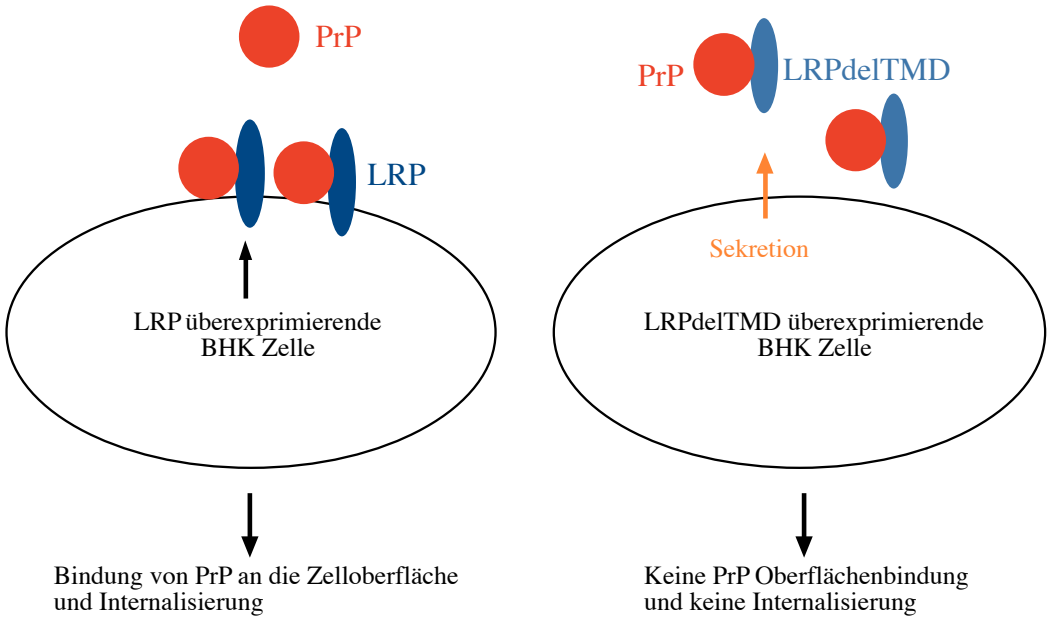


Abb.4 Modell zur Wirkungsweise der LRP Transmembran-Deletionsmutante LRPdelTMD. BHK Zellen, welche volle Länge LRP überexprimieren, binden und internalisieren das prion protein. Im Gegensatz dazu können BHK Zellen, welche LRPdelTMD in das Medium sezernieren kein prion protein binden und internalisieren. LRPdelTMD wird in das Medium sezerniert (*decoy*- oder *Lockvogel*- Effekt).

Weiterhin wollten wir wissen, ob die beobachetete erhöhte Aufnahme des Prion-Proteins einen aktiven Rezeptorvermittelten Prozess darstellt. Durch die Hemmung der Internalisierung des Prion-Proteins bei einer Inkubationstemperatur von 4°C konnte gezeigt werden, daß es sich bei der Aufnahme des Prion-Proteins tatsächlich um einen aktiven rezeptorvermittelten Prozess handelt (Gauczynski *et al.*, 2001b).

2.4 Eine Transmembran-Deletionsmutante (TMD) des Laminin-Rezeptors verhindert die Aufnahme von PrP

Der Laminin-Rezeptor besitzt eine vermeintliche Transmembrandomäne, welche sich von Aminosäure 86-101 erstreckt (Castronovo *et al.*, 1991b). Um die Bedeutung der Transmembrandomäne zu untersuchen, generierten wir eine LRP Deletionsmutante, bei der diese Region fehlte. Die PrP Bindedomänen des Rezeptors blieben bei dieser Deletion unberührt. Die Expression dieser Mutante in BHK Zellen durch Transfektion mit rekombinanter Semliki-Forest-Virus-(SFV)-RNA führte im Gegensatz zum Wildtyp-LRP-Protein zur Sekretion des mutierten Proteins (Gauczynski *et al.*, 2001b). Wurde nun von außen zelluläres Prion-Protein zugegeben, war es nicht in der Lage an die Zellen zu binden. Exogenes Prion-Protein wurde also bereits im Medium von der dort vorhandenen Laminin-Rezeptor Mutante gebunden und abgefangen (*decoy*- oder *Lockvogel*- Effekt) (Abb. 4). Dies zeigt, daß die LRP Transmembrandeletionsmutante die Aufnahme von PrP verhindern kann (Gauczynski *et al.*, 2001b).

3 Ausblick

Die vorgestellten Studien und neusten Vorschungsergebnisse stellen klar heraus, daß es sich bei dem 37 kDa/67 kDa Laminin-Rezeptor um den Rezeptor für das zelluläre Prion-Protein handelt. Die Rolle, welche beide Proteine und vor allem das Prion-Protein im endozytotischer Weg der Zelle spielen ist spekulativ. Dem Prion-Protein werden vielfältige Funktionen in der Zelle nachgesagt, wie zum Beispiel ein Rolle bei der Kupferbindung (Brown *et al.*, 1997; Jackson *et al.*, 2001), Superoxiddismutase Aktivität (Brown *et al.*, 1999), Lamininbindung (Graner *et al.*, 2000), Signaltransduktionsaktivtät (Mouillet-Richard, 2000) und eine antiapoptotische Aktivität (Bounhar *et al.*, 2001). Kürzlich wurde sogar eine Funktion im Nukleinsäuremetabolismus gefunden, wobei PrP eine ähnliche Funktion wie retrovirale Nukleokapsidproteine ausführen kann (Gabus *et al.*, 2001). Welche der vorgeschlagenen Funktionen nun wirklich die Funktion des Prion-Proteins ist oder ob es sich möglicherweise um ein multifunktionelles Protein handelt bleibt weiterhin Gegenstand der Prionenforschung.

Die Entdeckung des 37 kDa/67 kDa Laminin-Rezeptors als Rezeptor des Prion-Proteins könnte weiteren Einblick in die zellulären Verhältnisse und die Funktion von PrP geben.

CHAPTER III

INTERACTION OF PRION PROTEINS WITH CELL SURFACE RECEPTORS, MOLECULAR CHAPERONES, AND OTHER MOLECULES

published as:

Sabine Gauczynski, Christoph Hundt, Christoph Leucht and Stefan Weiss. (2001) Interaction of prion proteins with cell surface receptors, molecular chaperones, and other molecules. *Adv.Protein Chem.*,157, 319-325

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.*, 1995b). In addition, Harris observed that chicken PrP binds to the surface of mammalian cells via heparan sulfates on the cell surface (Shyng *et al.*, 1995a). 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 (Brimacombe *et al.*, 1999; Brown *et al.*, 1997), 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).

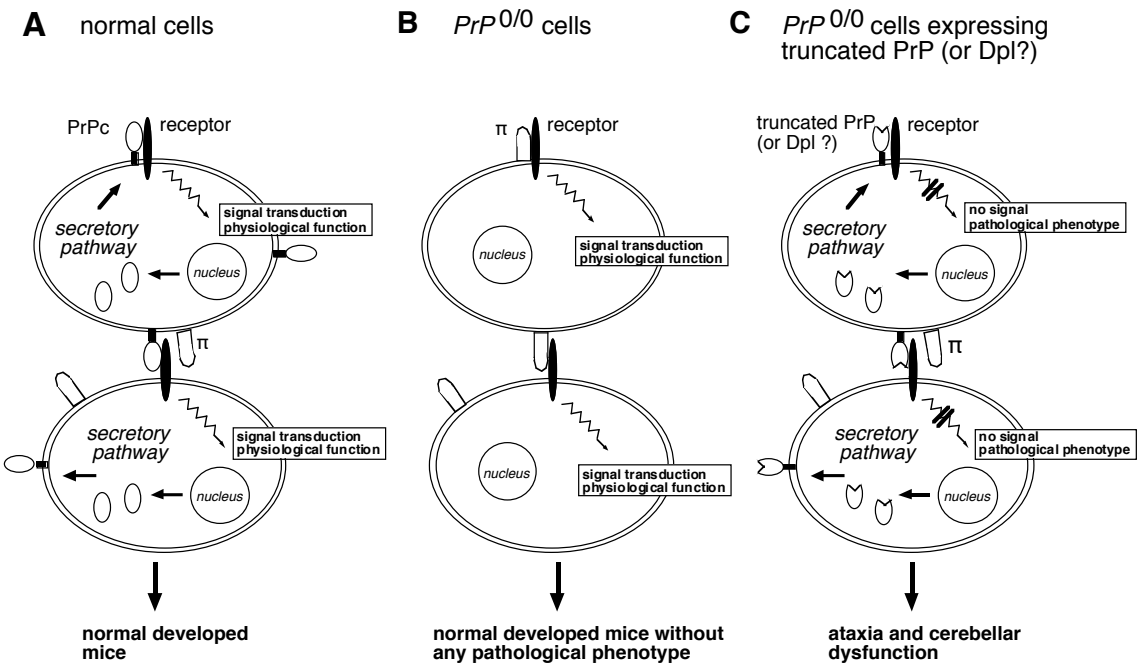


Fig. 1 Model of PrP^c- and receptor-mediated signal transduction. In the normal cell, PrP^c and receptor molecules from the same cell or from different cells can interact and promote signal transduction (A). The same signal might be elicited by the binding of a conjectural protein designated π, which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

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 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, 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.

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.

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.

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}

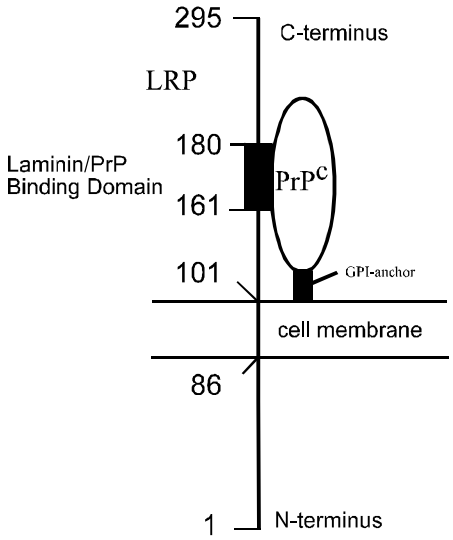


Fig. 2 Schematic view of the prion protein (PrP) and the 37 kDa laminin receptor precursor (LRP) on the surface of a scrapie-infectable cell. PrP is anchored by GPI (Blochberger *et al.*, 1997) and is thought to colocalize with LRP. The putative transmembrane region of LRP stretches from aa 86 to aa101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

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). 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.*, 1991a; 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 a (440 kDa), B1 or b, and B2 or g (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 □-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

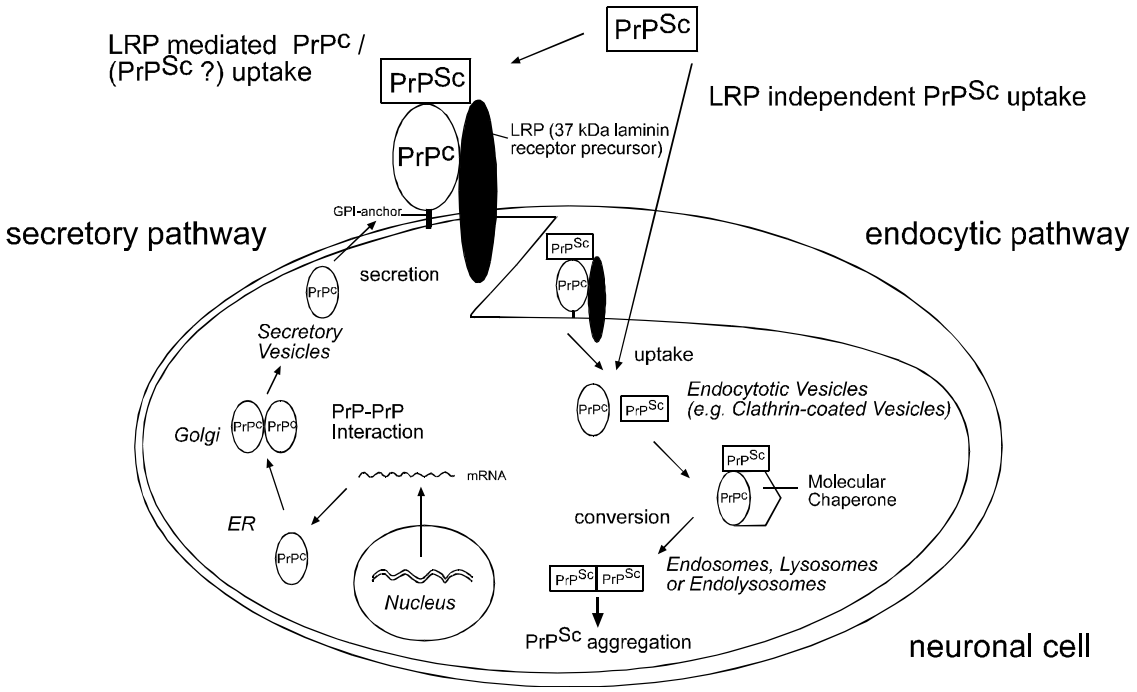


Fig.3 Model of the life cycle of prions. PrP^c is synthesized in the rough endoplasmatic reticulum (ER), and after passing through the secretory pathway including the Golgi and secretory vesicles, reaches the surface of a PrP^{Sc} infectable cell where it is anchored via a glycosylphosphatidyl inositol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

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.

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}.

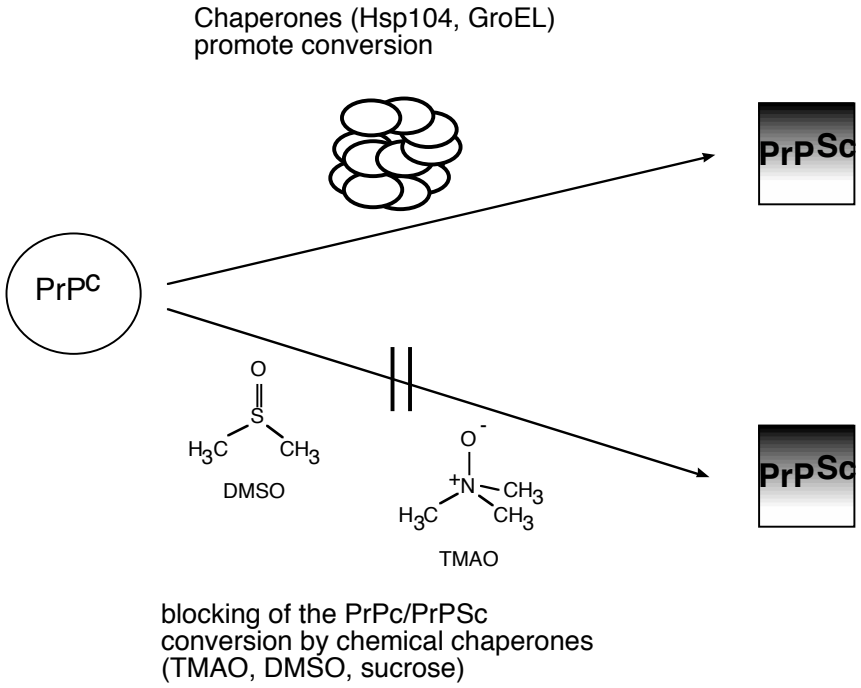


Fig.4 Influence of molecular and chemical chaperones on the conversion process of PrP^{c} to PrP^{Sc}. Molecular chaperones such as Hsp104 and GroEL promote the conversion reaction whereas the chemical chaperones TMAO, DMSO and sucrose prevent PrP^{Sc} formation.

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.

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 a-helix 2 and parts of a-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 a-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.

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 a-helical content in the presence of 0.2% SDS. The oligomerization process was then initiated by the reduction of the SDS-concentration. Formation of b-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.

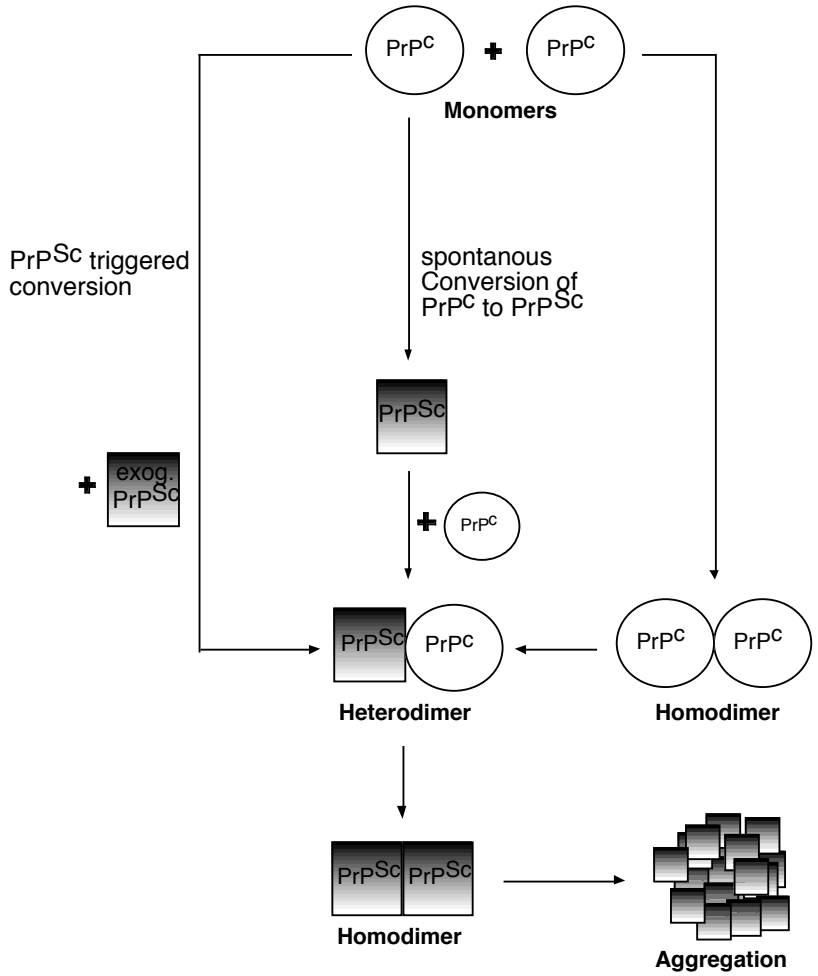


Fig. 5 Scheme of the conversion process of PrP^{c} to PrP^{Sc}. Three possibilities for the conversion of PrP^{c} into PrP^{Sc} do exist. An exogenous PrP^{Sc} triggers the conversion of PrP monomers leading directly to the hypothesized heterodimer consisiting of PrP^{c} and PrP^{Sc}. Genetic predisposition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

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 a-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 lgt11. 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 Ab 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.

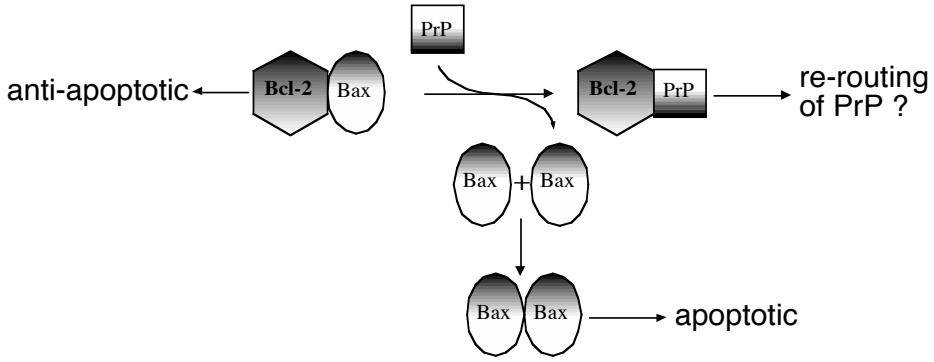


Fig. 6 Schematic view of Bcl-2::Bax, Bcl-2::PrP and Bax::Bax transitions and their possible role in cellular functions.

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 b-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 g-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 g-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

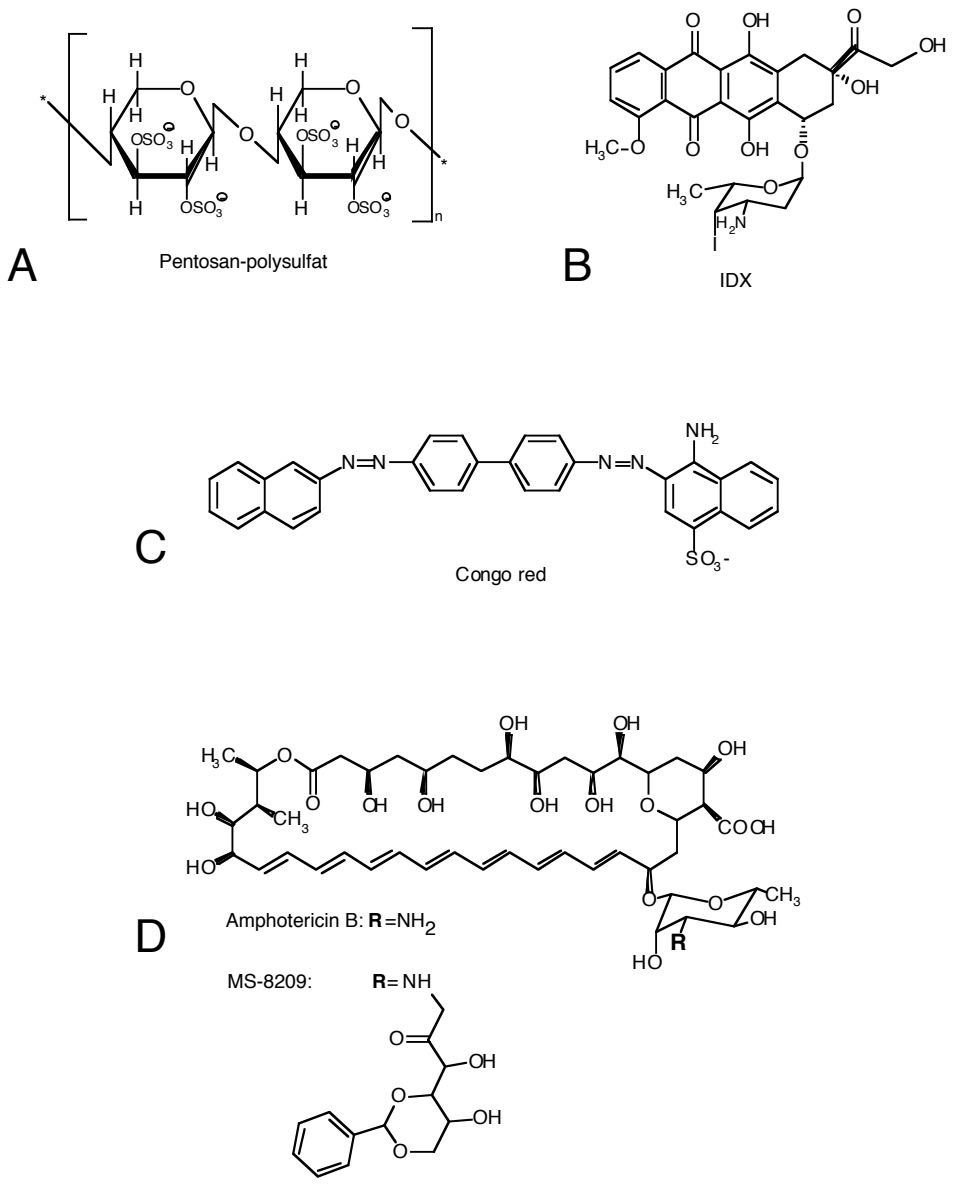


Fig. 7 Antiscrapie drugs of four different classes. (A) Pentosan polysulfate as a powerful drug belonging to the polyanion family. (B) IDX a derivative of doxorubicin. (C) Congo red belonging to the diazo dyes. (D) Amphotericin B and MS-8209 belonging to the family of polyene macrolide antibiotics.

after intracerebral infection (Adjou *et al.*, 1995; Demaimay *et al.*, 1994; McKenzie *et al.*, 1994; Pocchiari *et al.*, 1987; Xi *et al.*, 1992). 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 b-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 □-sheeted to a more □-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 IV

THE 37KDA/67KDA LAMININ RECEPTOR IS REQUIRED FOR PRP^{SC} PROPAGATION IN SCRAPIE-INFECTED NEURONAL CELLS

Published as:

Christoph Leucht, Steve Simoneau, Clémence Rey, Karen Vana, Roman Rieger, Corinne Ida Lasmézas and Stefan Weiss. (2003)

The 37kDa/67kDa laminin receptor is required for PrP^{Sc} propagation in scrapie-infected neuronal cells. *EMBO Reports*, **4**, 290–295.

Abstract

The accumulation of PrP^{Sc} in scrapie-infected neuronal cells has been prevented by three approaches: (i) transfection of ScMNB cells with an antisense laminin receptor precursor (LRP) RNA-expression plasmid, (ii) transfection of ScN2a and ScGT1 cells with small interfering RNAs (siRNAs) specific for the LRP mRNA, and (iii) incubation of ScN2a cells with an anti LRP/LR antibody. LRP antisense RNA and LRP siRNAs reduced LRP/LR expression and inhibited PrP^{Sc} accumulation these cells. The treatments also reduced PrP^{c} levels. The anti-LRP/LR antibody, W3, abolished PrP^{Sc} accumulation and reduced PrP^{c} levels after 7 days of incubation. Cells remained free of PrP^{Sc} after beeing cultured 14 additional days without the antibody, whereas the PrP^{c} level was restored. Our results demonstrate the necessity of the laminin receptor (LRP/LR) for PrP^{Sc} propagation in cultured cells and suggest that LRP/LR-specific antibodies as powerful therapeutic tools in the treatment of transmissible spongiform encephalopathies.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative disorders which includes Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep (Aguzzi and Weissmann, 1998; Lasmézas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann, 1999). The main pathogenic event in the development of TSEs is the conversion of PrP^{c}, the normal cellular form of the prion protein, to PrPSc. An important feature of PrP^{Sc} is its partial resistance to proteases, which makes it biochemically distinguishable from PrP^{c} (Caughey and Raymond, 1991). Recently, we identified the laminin receptor (LRP/LR) as the cell-surface receptor for the cellular prion protein (PrP^{c}) (Gauczynski *et al.*, 2001b). Heparan sulfate proteoglycans (HSPGs) have been shown to function as cofactors or co-receptors the binding of PrP^{c} to LRP/LR (Hundt *et al.*, 2001). The LRP/LR has been shown to interact directly with the prion protein in the yeast two-hybrid system (Rieger *et al.*, 1997). This interaction was confirmed by pull-down assays in cotransfected COS-7 cells and coinfected insect cells (Rieger *et al.*, 1997). Furthermore, increased levels of the LRP were found in the brain, spleen and pancreas of scrapie-infected mice and hamsters as well as in scrapie-infected neuroblastoma cells,

which are a well characterized *in vitro* model for scrapie infection (Rieger *et al.*, 1997). These data suggest a link between the LRP/LR and prion propagation.

The non-integrin LRP/LR laminin receptor is a multifunctional protein that is required for cell differentiation, movement and growth (for review see (Gauczynski *et al.*, 2001a)). Its cDNA encodes a 37-kDa precursor protein (LRP) also known as p40 and has been cloned from different species by several groups.This protein has been reported to be ribosome-associated, to bind to histones H2A, H2B and H4 and to be the precursor of the metastasis-associated 67 kDa mature high-affinity laminin receptor (LR) (for review (Gauczynski *et al.*, 2001a; Leucht and Weiss, 2002)). The 67-kDa LR is consistently upregulated in aggressive carcinoma suggesting a role in cell homeostasis and cohesion. The amino acid sequence of the receptor is highly conserved throughout evolution with at least 98.3% homology between mouse, human and bovine sequences and 99% homology between rat and human sequences (for review (Gauczynski *et al.*, 2001a; Leucht and Weiss, 2002)). Published data suggest the existence of at least six LR genes in the mouse genome; one of them is localised on chromosome 9 and at least two copies are thought to be functional (Douville and Carbonetto, 1992). Using TRIBE-MCL, an algorithm for the detection of protein families (Enright *et al.*, 2002), five LR genes were identified when the program was used to search the latest mouse draft genome sequence (Mouse Genome Sequencing Consortium, 2003, available at http://www.ensembl.org). The LRP gene on chromosome nine has seven exons and six introns, but in contrast to earlier results (Douville and Carbonetto, 1992) no LRP/LR gene on chromosome 6 has been identified. Interestingly, genes which affect susceptibility to prions have been identified on mouse chromosome nine (Stephenson *et al.*, 2000).

PrP specific antibodies have successfully been used in preventing prion propagation *in vitro* and *in vivo* as follows: first, the accumulation of PrP^{Sc} in scrapie-infected neuroblastoma cells was inhibited by PrP-specific antibodies (Peretz *et al.*, 2001); second, scrapie infection was abolished by transgenic expression of PrP specific antibodies in mice (Heppner *et al.*, 2001). The epitope recognized by the antibody that has the most potent effect on PrP^{Sc}, D18, consists od amino-acid residues 132-156 of PrP, which includes helix A (residues 144-154). Because PrP residues 144-179 have been shown to constitute a binding site for the LRP/LR (Hundt *et al.*, 2001), we investigated whether an antibody directed against the LRP/LR, the cellular receptor of PrP^{c} (Gauczynski *et al.*, 2001b), can also be used to interfere with the metabolism of PrP^{Sc}. To ablate LRP/LR expression from all putative LRP/LR-encoding genes, we used an

antisense RNA and a small interfering RNA (siRNA) approach. We investigated whether these strategies had an effect on prion propagation in several scrapie infected cell systems.

Results and Discussion

Antisense LRP mRNA prevents PrP^{Sc} propagation

To produce LRP antisense messenger RNA, we cloned a region of LRP complemantary DNA from nucleotide position –65 to 901 into the expression plasmid pCI-neo, to produce the pCI-neo-asLRP plasmid. After transient transfection of pCI-neo-asLRP into ScMNB cells, we confirmed antisense LRP RNA expression in these cells (Figure 1A). The level of LRP mRNA was greatly reduced 38 h after tranfection (Figure 1B). Using phosphoimaging, this reduction was quantified and LRP mRNA levels were found to be 80-85% of normal LRP mRNA expression levels. A similar reduction in target mRNA has been shown in other studies that have used the antisense RNA method to downregulate the expression of the myelin basic protein (Katsuki *et al.*, 1988) (80% reduction), and *Wnt-1* (Erickson *et al.*, 1993) (up to 98% reduction). At the level of protein expression, no LRP was detected by western blotting 48 h after transfection (Figure 1C). Analysis of the cells 72 h post transfection showed an absence of PrP^{Sc} propagation (Figure 1D) in cells with reduced LRP levels (Figure 1C). Levels of PrP^{Sc} were unaffected in cells transfected with the pCI-neo as compared with untransfected cells (Figure 1D). In ScMNB cells we were able to detect only the diglycosylated form of PrP using the SAF70 antibody, whereas in ScN2a and ScGT1 cells we observed the classic three-band pattern. We observed a reduction in PrP^{c} level which might be caused by an altered PrP^{c} metabolism. Previous data indicate that PrP^{c} internalization is strongly dependent on the presence of LRP/LR at the cell surface (Gauczynski *et al.*, 2001b), where LRP/LR binds to PrP^{c} via two distinct binding domains: the octapeptide region and the region encompassing amino acids 144 to 179 (Hundt *et al.*, 2001). This is consistent with a very recent study, in which it was found that the octarepeat region is essential for internalization of PrP^{c} (Nunziante *et al.*, 2002). Hence, the altered PrP^{c} level is likely to be due to a perturbed metabolism of the protein.

LRP-specific siRNAs prevent PrP^{Sc} propagation

SiRNAs were used to verify the results obtained using the LRP antisense RNA construct. This method has been used succesfully in other studies to knock-down target gene expression levels (Elbashir *et al.*, 2001). We tested four different LRP-specific siRNAs for their ability to repress LRP expression in ScN2a cells. All of them repressed LRP/LR synthesis (Figure 2A). Figure 2B shows data from a timecourse experiment carried out to analyse the effect of siRNA-LRP3 on PrP^{Sc} propagation in ScN2a cells. Seventy-two hours after transfection PrP^{Sc} propagation was completely abolished by siRNA-LRP3, whereas siRNA-LRP1, siRNA-LRP4 and a control siRNA (lamin A/C, described in Elbashir *et al.*, 2001) had a smaller effect (siRNA-LRP1+ siRNA-LRP4) or no effect (control) on PrP^{Sc} levels. PrP^{c} levels were reduced in the presence of siRNA_LRP 3. The same effects were observed with LRP antisense RNA 72 h after transfection. In contrast to PrP^{Sc}, PrP^{c} levels increased 96 hours after transfection probably due to a decrease in siRNA effectiveness with time.

We also tested the efficiency of the reduction of LRP expression using siRNAs in ScGT1 cells showing a robust PrP^{Sc} phenotype (that is, these cells propagate PrP^{Sc} over a long period of time). The results were consistent with those obtained using ScN2a cells, with a strong reduction of PrP^{Sc} correlated with LRP downregulation (Figure 2C).

Anti-LRP/LR antibody W3 prevents PrP^{Sc} accumulation

LRP/LR specific antibodies have been used successfully to compete with recombinant prion protein for binding to the LRP/LR in different mammalian cell types (Gauczynski *et al.*, 2001b), showing that the LRP/LR has a crucial role in the metabolism of PrP^{c}. Using the LRP/LR-specific antibody, W3, (Rieger *et al.*, 1997) in ScN2a cells and observed a reduction of PrP^{Sc} to undetectable levels (Figure 3A, B). The antibody was used at different concentrations of 6-64 $\mu g\ ml^{-1}$. At a concentration of 12 $\mu g\ ml^{-1}$ a reduction in PrP^{Sc} level was observed. At a higher concentration (64 $\mu g\ ml^{-1}$), PrP^{Sc} accumulation was totally abolished after incubation for three days indicating a dose dependent effect (Figure 3A). In a timecourse experiment, we found a complete clearance of PrP^{Sc} after incubation for one week, using an antibody concentration of 32$\mu g\ ml^{-1}$ (Figure 3B). These results are concistent with a previous study, in which different anti-PrP antibodies were used to reduce PrP^{Sc} levels in cultured cells

(Peretz *et al.*, 2001; Table I). In that study, PrP antibody concentrations of 1.2-10 μg ml^{-1} were sufficient to clear PrP^{Sc} from ScN2a cells after one week of incubation (Table I).
We also incubated ScN2a cells in which PrP^{Sc} had been previously cleared by W3 for a further two weeks without any antibody, and showed that no PrP^{Sc} reappeared (Figure 3B). PrP^{c} levels in W3-treated cells were reduced after 7 days of W3 antibody incubation and totally restored after a further two-week incubation in the absence of the antibody (Figure 3B).

Table I Efficacy of anti-PrP and anti-LRP/LR antibodies in clearance of PrP^{Sc} from ScN2a cells

Antibody	anti-LRP/LR	anti-PrP[1]			
Incubation time	1 week	1 week			
Antibody	W3	D18	D13	R1	R2
Effective Concentration [μgml^{-1}]	32	1.2	2.5	10	10

[1] data taken from (Peretz *et al*., 2001)

Role of LRP/LR in PrP^{Sc} propagation in cultured cells

The knock down of LRP/LR on the cell surface by LRP antisense RNAs or siRNAs, and the blockage of LRP/LR binding sites by the W3 anti-LRP/LR antibody are most likely to interfere with PrP levels by blocking the PrP internalization process. However, some PrP^{c} can still be synthesized and transported via the secretory pathway to the cell surface (Figures 1D, 2B, 3). Conversion of PrP^{c} into PrP^{Sc} is thought to take place either at the cell membrane or in the endocytic pathway. Thus, it is possible that due to the lack of PrP^{c} within the endocytic pathway no PrP^{Sc} can be formed resulting in a time dependent reduction of PrP^{Sc} (Figure 2B, 3B). It is also possible that that the LRP/LR has a function in the conversion of PrP^{C} to PrP^{Sc} and that the abcense of LRP/LR from the cell surface affects PrP^{Sc} formation. PrP^{Sc}

propagation cannot be restored after cessation of incubation with anti-LRP/LR antibody (Fig. 3B) due to the absence of any PrP^{Sc} to re-initiate the conversion process. In contrast, PrP^{c} levels were completely restored after cessation of incubation with the anti-LRP/LR antibody (Fig. 3B). Furthermore, depletion or blockage of LRP/LR on the cell surface might directly prevent PrP^{Sc} binding and internalization. In summary our results show, that the LRP/LR is not only involved in PrP^{c} metabolism, as demonstrated in previous reports (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001), but also plays a central role in prion propagation. The fact that LRP/LR specific antibodies are able to clear PrP^{Sc} from neuroblastoma cells provides possibilities for the development of new experimental therapies for TSEs..

Methods

Construction of pCI-neo-asLRP. Base –65 to 901 of the LRP cDNA were amplified by PCR with reverse transcription (RT-PCR) from total RNA isolated from N2a cells, introducing the restriction sites *Nhe*I and *Sma*I. The LRP cassette was cloned via *Nhe*I/*Sma*I in antisense orientation into the plasmid pCI-neo, resulting in pCI-neo-asLRP.Cloning was confirmed by sequencing.

Cell culture. ScMNB and ScN2a cells (both lines are neuroblastoma cells chronically infected with scrapie) were grown in DMEM, 10% fetal bovine serum, 2mM Glutamax, 100 units/ml penicillin and 10 $\mu g ml^{-1}$ streptomycin sulfate, at 37°C with 5% CO_2. ScN2a cells were produced as described previously (Bosque and Prusiner, 2000). The ScGT1-7 cells (GT1 hypothalamic neuronal cells chronically infected with the Chandler scrapie isolate) were provided by S. Lehmann, and were cultured as previously described (Mange *et al.*, 2000) with the exception that Dulbbecco's modified Eagles medium (DMEM) was replaced with Opti-MEM (Gibco Life Sciences).

Inhibition studies using the W3 antibody. ScN2a cells ($1x10^{6}$) were incubated in normal growth medium (DMEM, 10% fetal bovine serum, 2mM Glutamax) supplemented with the purified polyclonal anti-LRP/LR antibody, W3, at varying concentrations. After incubation the cells were harvested, lysed and analyzed by western blotting.

Inhibition studies using LRP antisense RNA. ScMNB cells were grown in a six-well plate to 60% density. The cells were transfected with pCI-neo-asLRP and pCI-neo (control plasmid) using Lipofectamin (Invitrogen) according to the manufacturer's instructions.

Transfection efficiencies were determined using a chloramphenicol acetyltransferase construct, and were estimated to be approximatlely 80% on average (data not shown). Cells were harvested 72 h after transfection, lysed and analysed by western blotting.

siRNA inhibition studies. Four different pairs of complementary 21-nucleotide RNAs corrosponding to regions of the LRP cDNA were made (Ambion). As a control the lamin A/C RNA duplex was used (Elbashir *et al.*, 2001). The single stranded complementary RNAs were annealed in annealing buffer (provided by the manufacturer) for 1 min at 90 °C, followed by 1 h at 37 °C. The RNA duplexes were transfected into ScN2a cells (cultured in Opti-MEM medium, Invitrogen) using Oligofectamin (Invitrogen) in accordance with the manufacturer's instructions. ScGT1-7 cells were seeded in 60-mm petri dishes (5 x 10^5 cells per dish) and transfected the following day with 10µg of each 21-nucleotide RNA pairs using Exgen 500 (Fermentas) in accordance with the manufacturer's instructions.

Ribonuclease protection assays. Total RNA was purified from transfected ScMNB cells and used in a Ribonuclease Protection Assay (RPA) using the RPA III kit (Ambion). An antisense riboprobe was made by *in vitro* transcription from pCI-neo-asLRP, following linearization of the plasmid with *Eco*RI, in the presence of (α-^{32}P)-UTP. The antisense riboprobe was combined with the total RNA and the mixture was then precipitated. The precipitates were dissolved in hybridization buffer, denatured and hybridized with the total RNA. This was followed by incubation with RNAse for 30 min at 37°C, followed by inactivation of the RNAse and ethanol precipitation of the RNA. Protected RNA fragments were separated on a 5% acrylamid/urea gel and visualized using a Storm 860 phosphorimager equipped with ImageQuant software.

RT-PCR. Total RNA was purified from transfected ScMNB cells and cDNA synthesis was carried out using an oligo(dT) primer in an RT-reaction. The resulting cDNA was then amplified by PCR using a 5´-oligodeoxyribonucleotide corrosponding to a sequence in the 3´-end of the cytomegalovirus promoter and a 3´-oligodeoxyribonucleotide corrosponding to a sequence in the 5´-region of the simian virs 40 polyadenylation signal. PCR products were separated on a 1% agarose gel and stained withethidium bromide.

Western blotting. Cytoplasmic lysats were made using a buffer containing 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5 % Triton X-100, and 0.5 % sodium desoxycholate. After centrifugation, the total protein content of the lysats was measured (BCA-Protein Assay, Pierce) and equal amounts of protein from each lysate were analysed. For PrP^{Sc} detection, cell

lysats were digested with proteinase K (20 µg ml^{-1}) for 1 h at 37°C. The reaction was stopped by addition of Pefabloc (1 mM) and the proteins were denatured with 6 M guanidine hydrochloride. The samples were boiled in SDS sample buffer and analyzed on an SDS polyacryamid gel containing 12.5% acrylamid. For PrP^{c} or PrP^{Sc} detection (from ScN2a cells) 10% Bis-Tris gels with MES running buffer (NuPAGE, Invitrogen) were used. Proteins were blotted on a polyvinylidene difluoride membrane, blocked and incubated overnight with the monoclonal antibody SAF70/SAF32/SAF84 (diluted 1:5000 in blocking solution) or A7 (diluted 1:2500 in blocking solution) for PrP detection. The polyclonal anti LRP/LR antibody W3 (Rieger *et al.*, 1997) (1:2000) or the monoclonal antibody 43512 (1 µg ml^{-1}) for LRP detection or anti-□ actin antibody (Chemicon) (1:5000) for □-actin detection. After washing with TBS/0.05% Tween 20 the blot was incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma) (1:2500). Detection was carried out by enhanced chemiluminescence (Western Lightning, NEN).

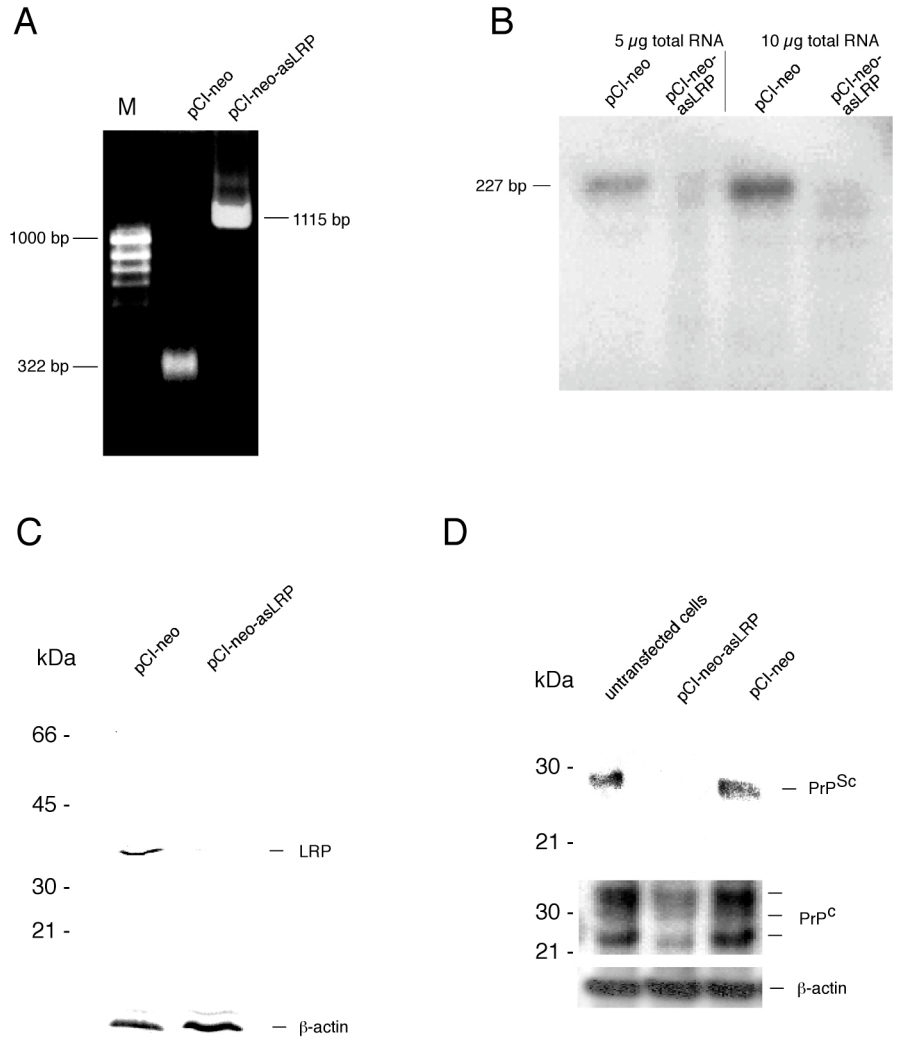


Fig. 1 Abolition of PrP^{Sc} propagation using laminin receptor precursor (LRP) antisense RNA. **(A)** Analysis by PCR with reverse transcription of total RNA extracts of transfected ScMNB cells. Oligodesoxythymidine-primed complementary DNA was amplified by PCR using specific primers for the pCI-neo plasmid. This gave a 322-bp cDNA fragment for the pCI-neo transfected cells and a 1115-bp cDNA fragment for the pCI-neo-asLRP transfected cells. **(B)** A ribonuclease protection assay was carried out on total RNA from cells transfected with either pCI-neo or pCI-neo-asLRP; the RNA was then separated on a 5% acrylamid/urea gel. 5 µg and 10 µg of total RNA was used and in both cases the level of LRP messenger RNA was reduced by 80-85% in cells transfected with pCI-neo-asLRP (quantified with posphorimaging). **(C)** Western blot analysis of cell lysats from pCI-neo and pCI-neo-asLRP transfected ScMNB cells assayed 48 hours after transfection. LRP was detected using the polyclonal anti-LRP/LR antibody, W3. β-actin was detected using an anti-β-actin antibody as loading control. **(D)** ScMNB cells were transfected with pCI-neo and pCI-neo-asLRP. The PrP^{Sc} content of ScMNB cells was determined 72 h after transfection. The monoclonal anti-PrP antibody SAF70 was used for PrP^{Sc} detection and the SAF32 antibody was used for dtection of PrP^{C}.

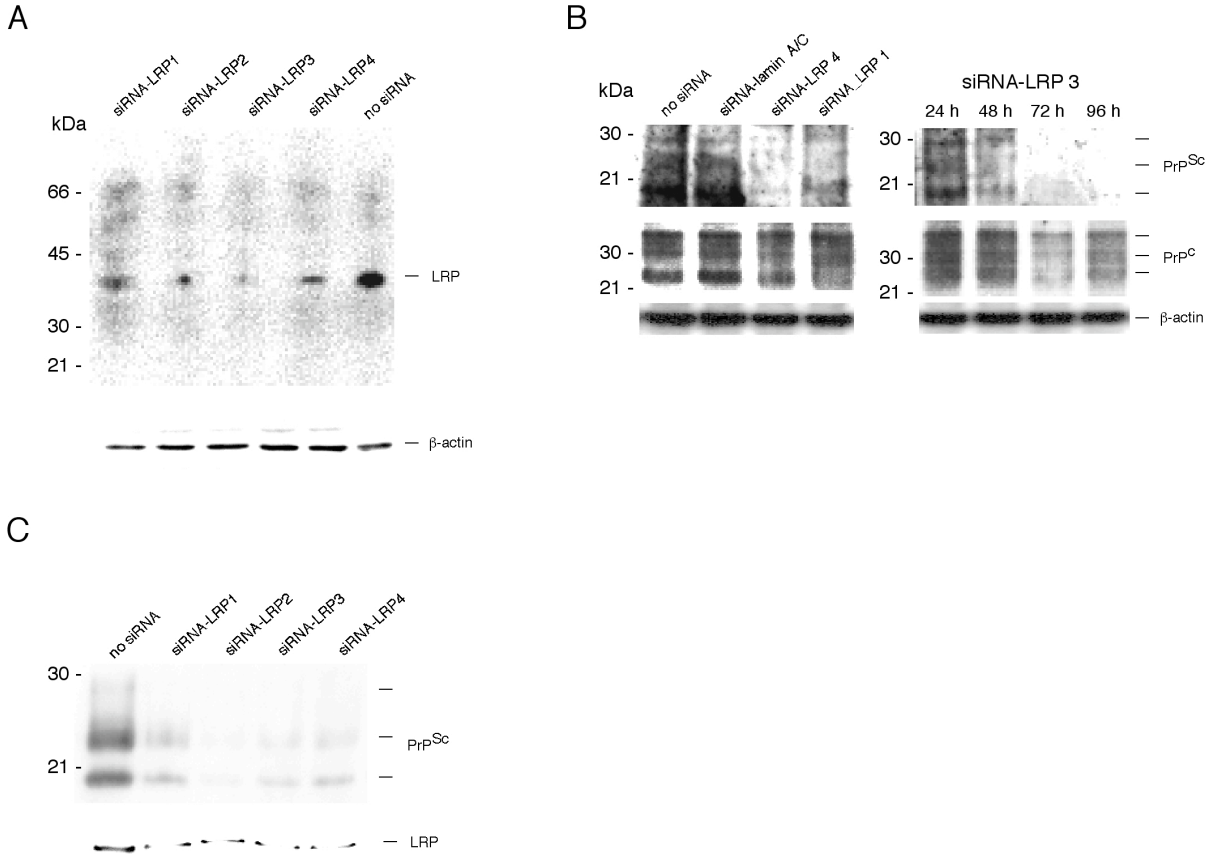


Fig. 2 Inhibition of PrP^{Sc} propagation using small interfering RNAs (siRNAs). **(A)** Western blot analysis of ScN2a cells transfected with siRNAs. Cells were analysed 72 hours after transfection using the polyclonal anti-laminin receptor (LRP/LR) antibody W3. **(B)** The effect of siRNAs on PrP^{Sc} propagation was assayed 72 hours after transfection (left panel). The time dependent effect of siRNA-LRP3 on PrP^{Sc} propagation (right panel) was analysed using the SAF70 antibody; PrP^{c} was detected with the SAF32 antibody. -actin was detected using an anti--actin antibody as loading control. **(C)** Western blot analysis of siRNA-transfected ScGT1 cells at 72 hours after transfection. The cells were analysed using the mAb LR43512 (lower pannel) and the mAb SAF84 (upper pannel). All samples were normalized to equal protein concentrations.

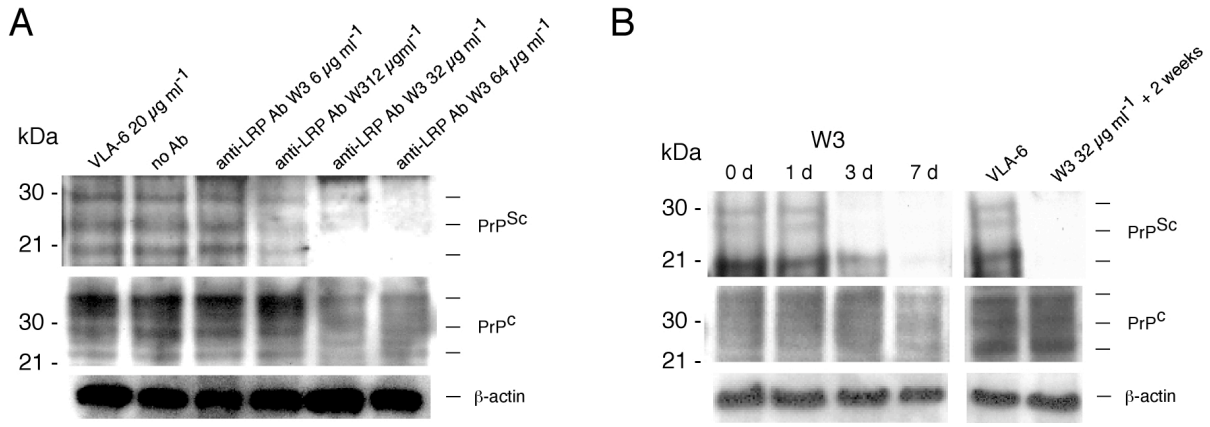


Fig. 3 The effect of the W3 anti-laminin receptor (LRP/LR) antibody on PrP^{Sc} propagation. **(A)** ScN2a cells were incubated with W3 at varying concentrations. The PrP^{Sc} content was determined after a 72 h incubation with the antibody W3. An anti VLA-6 (integrin-type laminin receptor) antibody was used as control. PrP^{Sc} was detected using the A7 polyconal antibody; PrP^{c} was detected with the SAF32 antibody. **(B)** ScN2a cells were incubated with W3 at 32µg/ml for varying durations. The last lane shows W3-treated ScN2a cells after an additional 2-week incubation without any antibody. PrP^{Sc} was detected with the SAF 70 antibody, PrP^{c} was detected with the SAF32 antibody. β-actin was detected using an anti-β-actin antibody as loading control.

CHAPTER V

THE 37-KDA/67-KDA LAMININ RECEPTOR ACTS AS THE CELL SURFACE RECEPTOR FOR THE CELLULAR PRION PROTEIN

published as:

Sabine Gauczynski, Jean-Michel Peyrin, Stéphane Haïk, Christoph Leucht, Christoph Hundt, Roman Rieger, Susanne Krasemann, Jean-Philippe Deslys, Dominique Dormont, Corinne Ida Lasmézas and Stefan Weiss. 2001.

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

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, 1991b; 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.

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.

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 82-88). pSFV-1 (Liljestrom and Garoff,

1991b), 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, $PrP^{0/0}$ 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 subsequently to the figures (see pages 122-128).

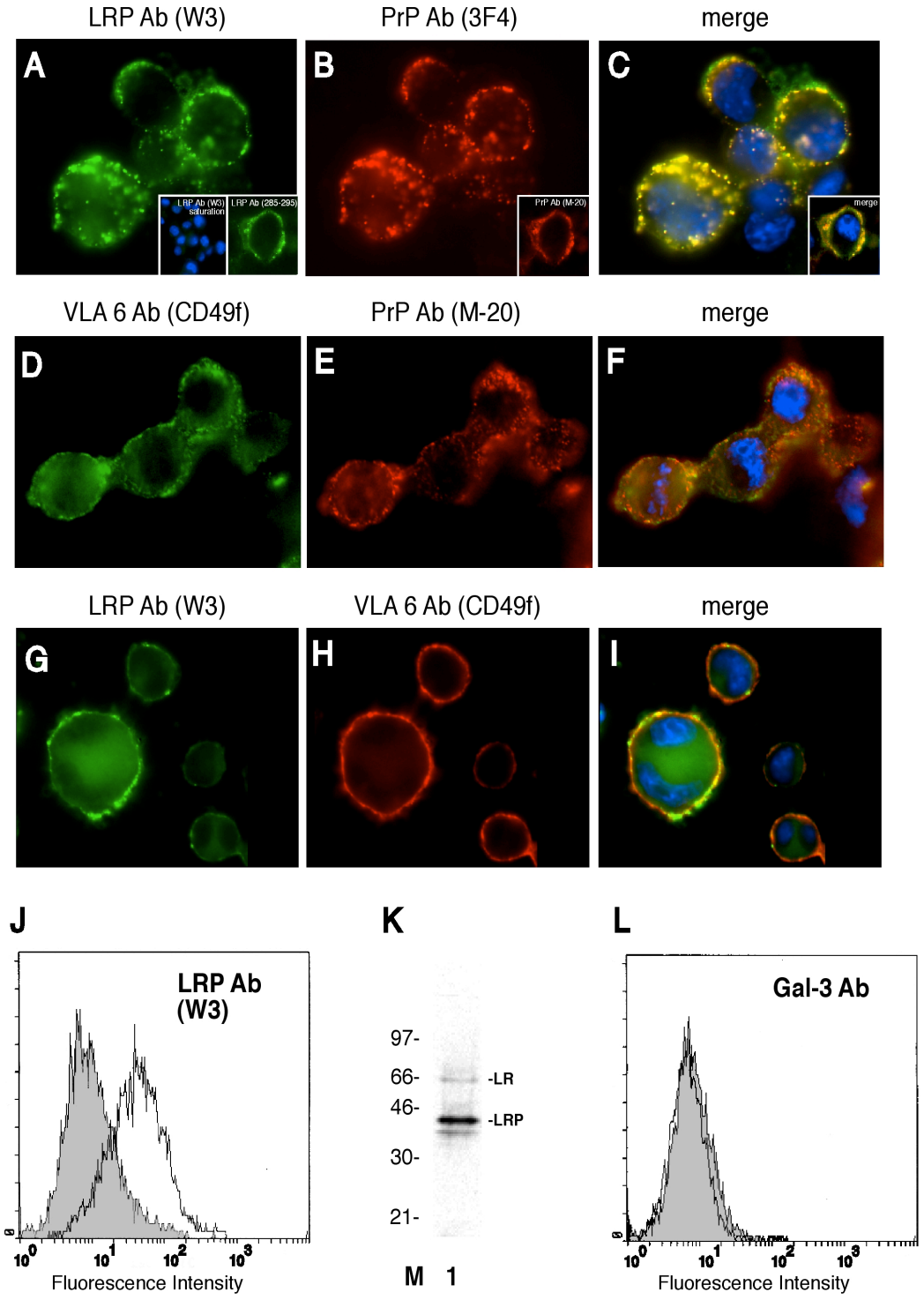


Fig. 1 Plasmamembrane-associated LRP/LR and PrP co-localize on the surface of neuroblastoma cells. Non-permeabilized N2a [MHM2] cells were incubated with the pAb LRP W3 [sec. Ab fluorescein isothiocyanate (FITC)] (**A**), pAb LRP W3 saturated with rec. GST::LRP [sec. Ab carbocyanine Cy2, 4□ 6-diamidine-2-phenylindole (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).

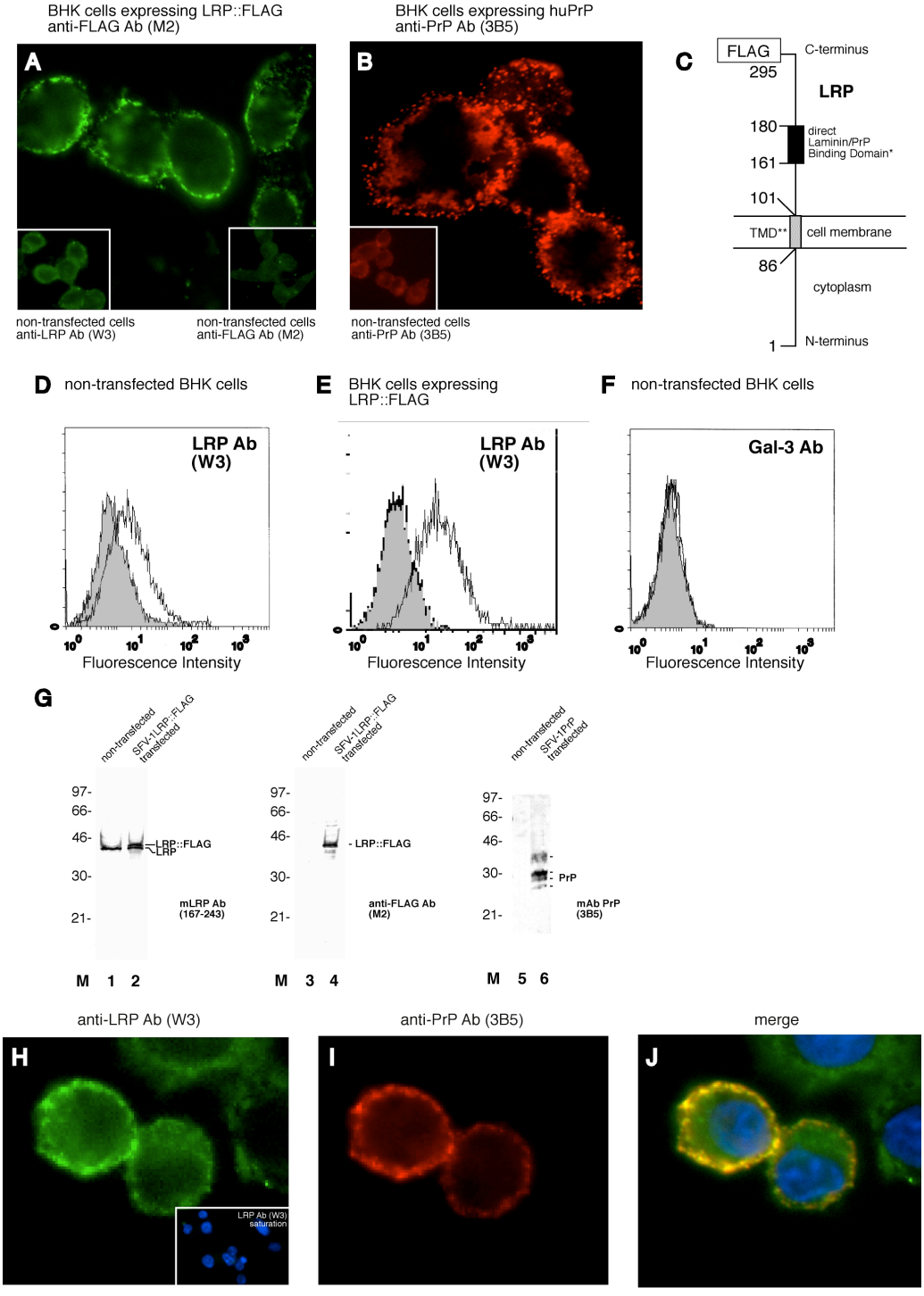
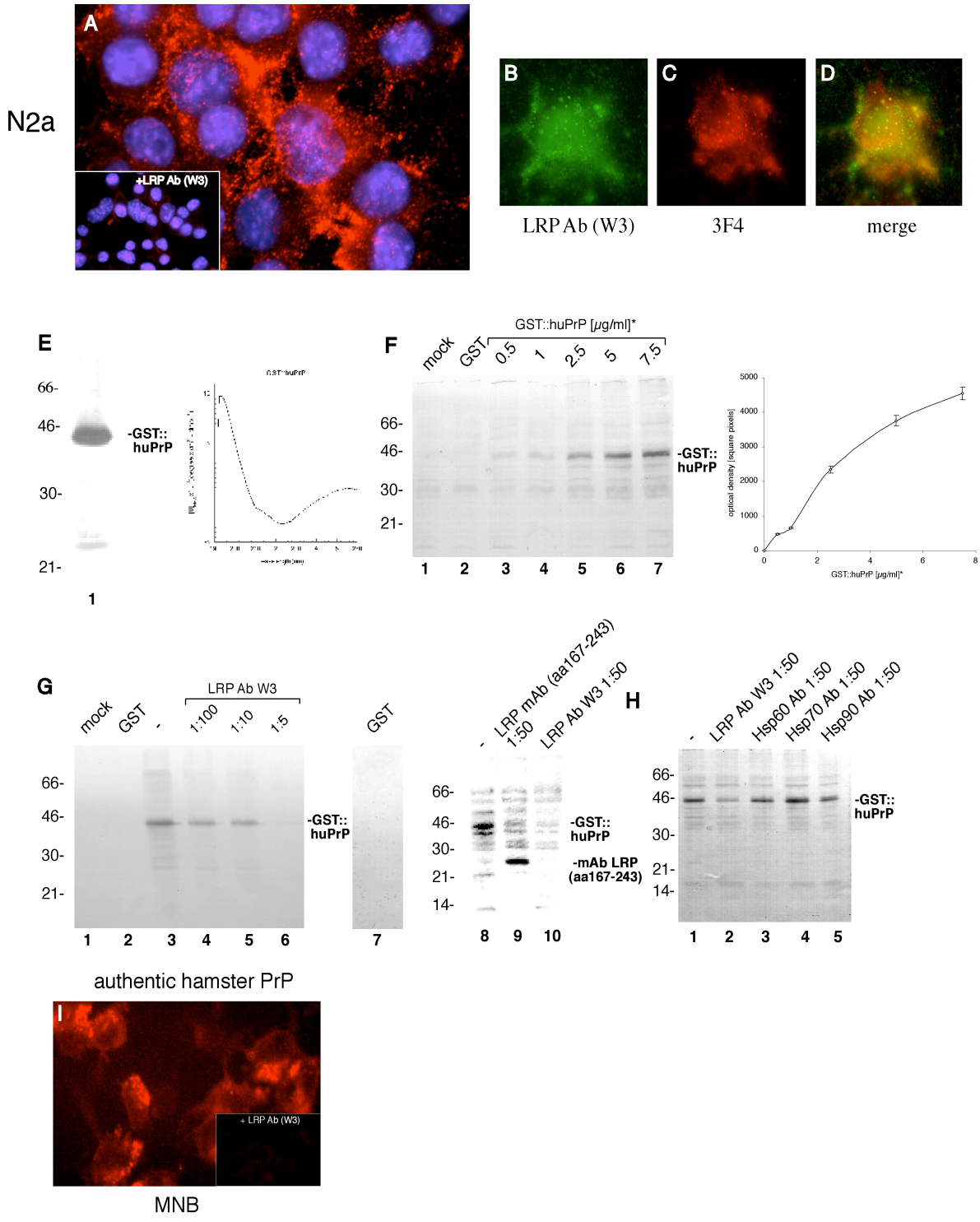
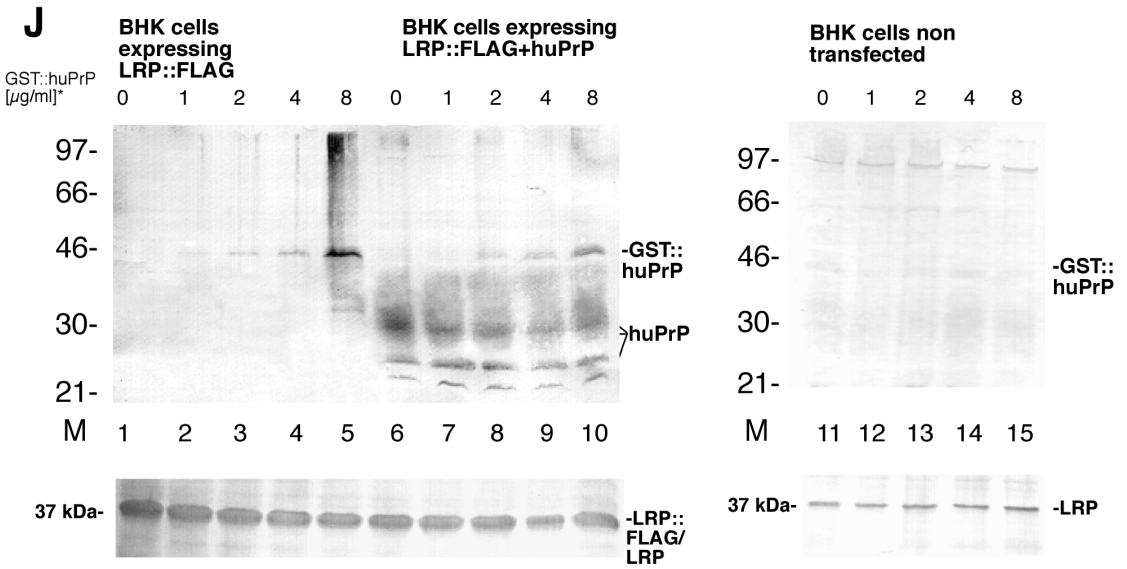
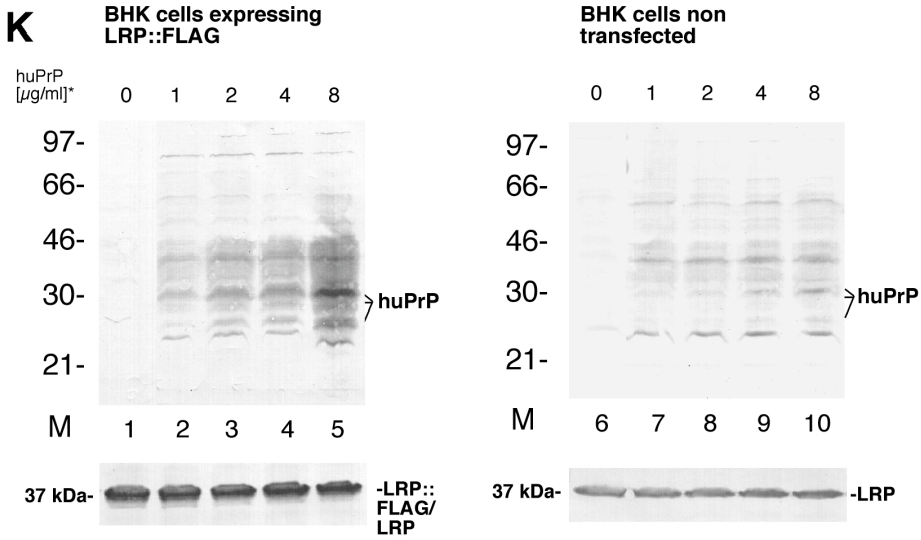


Fig. 2 Orientation, localization of LRP::FLAG and PrP and co-localization of both proteins in BHK cells transfected with rec. SFV RNAs. (**A**) Immunolocalization of LRP::FLAG to the cell membrane of non-permeabilized BHK cells transfected with rec. SFV LRP-FLAG RNA. Subcellular location was determined 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
mock
GST
-
LRP Ab W3
1:100
1:10
1:5
GST
66-
46-
30-
21-
-GST::
huPrP
1 2 3 4 5 6
7
authentic hamster PrP
H
-
LRP mAb (aa167-243)
1:50
LRP Ab W3 1:50
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-
46-
30-
21-
14-
-GST::
huPrP
1 2 3 4 5
I
+ LRP Ab (W3)
MNB


J
BHK cells expressing LRP::FLAG
BHK cells expressing LRP::FLAG+huPrP
BHK cells non transfected
GST::huPrP [μg/ml]*
0 1 2 4 8 0 1 2 4 8
0 1 2 4 8
97-
66-
46-
30-
21-
-GST:: huPrP
huPrP
M 1 2 3 4 5 6 7 8 9 10
M 11 12 13 14 15
37 kDa-
-LRP:: FLAG/ LRP
-LRP


K
BHK cells expressing LRP::FLAG
BHK cells non transfected
huPrP [μg/ml]*
0 1 2 4 8
0 1 2 4 8
97-
66-
46-
30-
21-
huPrP
M 1 2 3 4 5
M 6 7 8 9 10
37 kDa-
-LRP:: FLAG/ LRP
-LRP

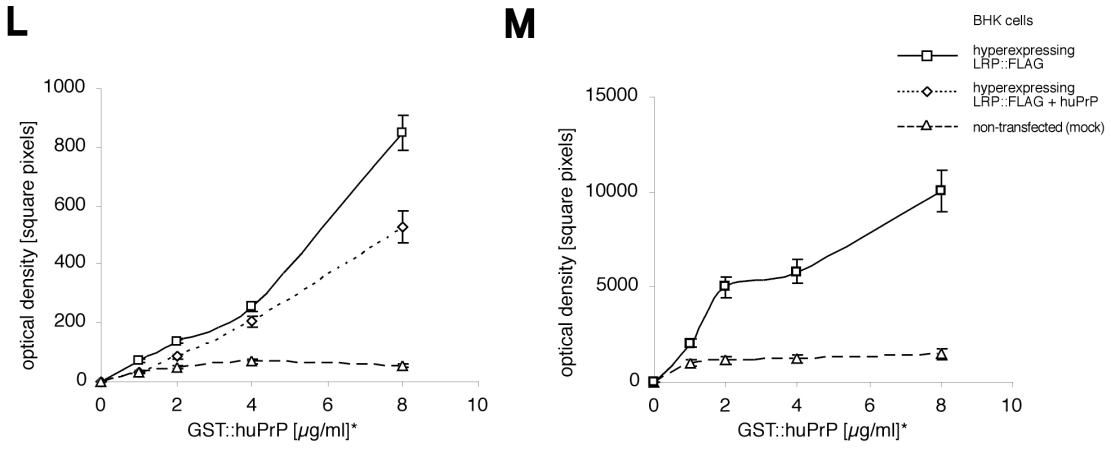

L
optical density [square pixels]
1000
800
600
400
200
0
0 2 4 6 8 10
GST::huPrP [μg/ml]*
M
BHK cells
hyperexpressing LRP::FLAG
hyperexpressing LRP::FLAG + huPrP
non-transfected (mock)
15000
10000
5000
0
0 2 4 6 8 10
GST::huPrP [μg/ml]*

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).

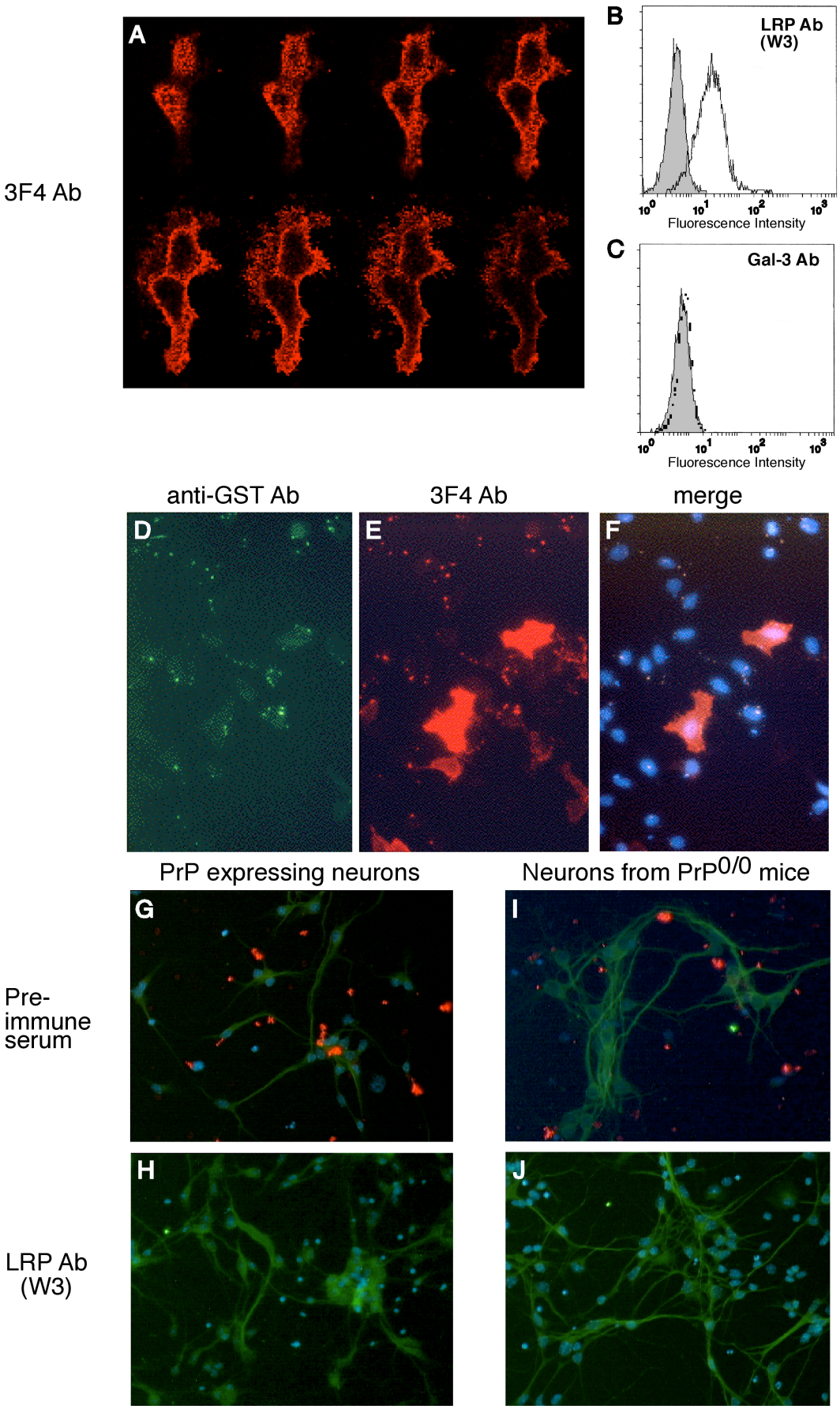

3F4 Ab
A
B
LRP Ab
(W3)
Fluorescence Intensity
C
Gal-3 Ab
Fluorescence Intensity
anti-GST Ab
3F4 Ab
merge
D
E
F
PrP expressing neurons
Neurons from PrP0/0 mice
Pre-
immune
serum
G
I
LRP Ab
(W3)
H
J

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).

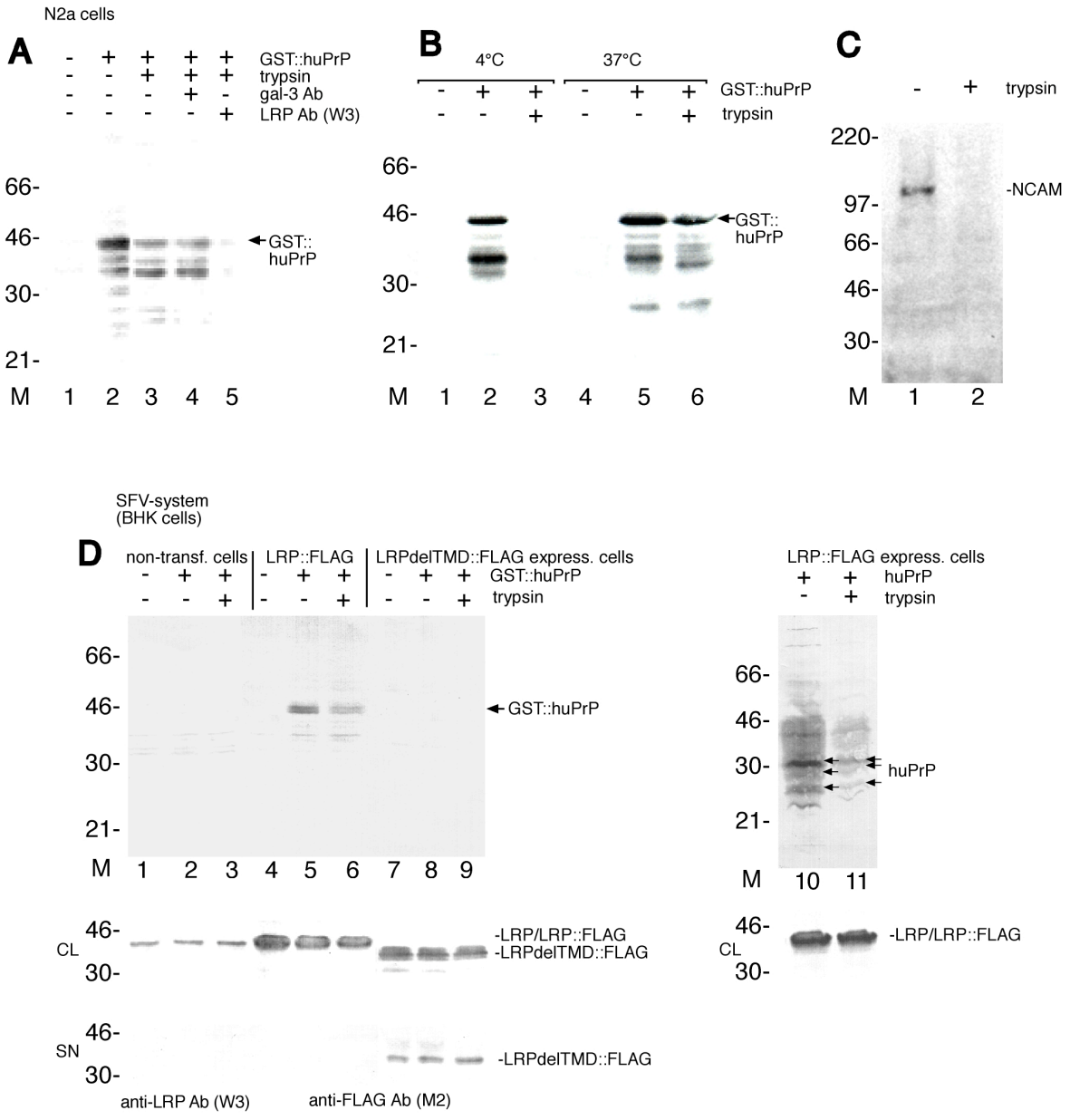


Fig. 5 LRP/LR-dependent binding and internalization of GST::huPrP by N2a cells. An LRP mutant lacking the transmembrane domain (LRPdelTMD) totally abolishes PrP binding and internalization on BHK cells. (**A**) Internalization of GST::huPrP by N2a cells. N2a cells not pre-incubated with antibodies (lane 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:

Supplementary Material section (SMS) for⊡

Gauczynski, S., Peyrin, J.-M., Haïk›, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J.-P., Dormont, D., Lasmézas, C. I. and Weiss, S. 2001.

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, 1991a) 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, 1997 #1919}, 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 VI

DIFFERENT ISOFORMS OF THE NON-INTEGRIN LAMININ RECEPTOR ARE PRESENT IN MOUSE BRAIN AND BIND PRP

Published as:

Steve Simoneau, Stéphane Haïk, Christoph Leucht, Dominique Dormont, Jean-Philippe Deslys, Stefan Weiss and Corinne Lasmézas. 2003

Different Isoforms of the Non-Integrin Laminin Receptor are Present in Mouse Brain and Bind PrP (2003). *Biological Chemistry*, **384**, 243–246

Abstract

The prion protein (PrP) plays a central role in prion diseases and identifying its cellular receptor appears to be of crucial interest. We showed in the yeast two hybrid system that PrP interacts with the 37 kDa precursor (LRP) of the high affinity 67 kDa laminin receptor (LR) which acts as the cellular receptor of PrP in cellular models. However, within the various isoforms of the receptor that have been identified so far, those which are present in the central nervous system and which bind PrP are still unknown. In this study, we have purified mouse brain fractions enriched in the laminin receptor and have performed overlay assays in order to identify those isoforms which interact with the prion protein. We show *i*) the presence, in mouse brain, of several isoforms of the LRP/LR corresponding to different maturation states of the receptor (44, 60, 67, and 220 kDa) and *ii*) the binding of all of these isoforms to PrP. Our data strongly support the physiological role of the laminin receptor/PrP interaction in the brain and highlight its relevance for TSE pathologies.

A fundamental event in the pathogenesis of human and animal prion diseases is the conformational modification of a normal host-encoded protein (PrP^{C}), from a soluble form to an aggregated, partially protease resistant form termed PrP^{Sc} enriched in β-sheeted structures (for review see (Prusiner *et al.*, 1998)). The misfolded isoform (PrP^{Sc}) of the prion protein accumulates in the central nervous system and in other areas, such as the lymphoreticular system, during the development of the disease. PrPsc is thought to be a major component of the causative agent of transmissible spongiform encephalopathies, also called prion diseases. Molecules interacting with PrP (for review see (Gauczynski *et al.*, 2001a)) which could play a role in the replication of the infectious particle, as well as the precise location where the conversion from PrP^{C} to PrP^{Sc} take place have to be identified (Caughey *et al.*, 1991; Telling *et al.*, 1995). It has been shown that the normal isoform, PrP^{C}, plays a central role in prion diseases: *i*) PrP knockout mice are resistant to prion infection (Bueler *et al.*, 1993) *ii*) when transgenic mice expressing different levels of PrP^{C} are infected with the agent of prion diseases, the duration of the incubation period is inversely proportional to the level of PrPc expressed *iii*) the PrP gene of the host controls the species barrier (Prusiner, 1993; Scott *et al.*, 1989) and *iv*) PrP^{C} expression is necessary for prion-induced neurodegeneration (Brandner *et al.*, 1996). Thus, characterizing the cellular receptor for the prion protein appears to be of crucial interest for understanding the mechanisms of prion replication, CNS invasion, and neurodegeneration characteristically linked to prion diseases. In the yeast two hybrid system, we have identified the 37 kDa precursor (LRP) of the 67 kDa laminin receptor (LR) as a protein which interacts directly with PrP^{C} (Rieger *et al.*, 1997). Coexpression of both LRP and PrP in insect and mammalian cells has confirmed this interaction (Rieger *et al.*, 1997). Furthermore, the level of LRP, which has been described previously as a receptor for the Sindbis virus on mammalian cells (Wang *et al.*, 1992), is increased in organs that support prion replication and PrP^{Sc} accumulation in experimental scrapie or bovine spongiform encephalopathy infected animals (Rieger *et al.*, 1997). In cellular models including primary cultures and neuronal cell lines, we demonstrated that LRP acts as the cellular receptor for PrPc, mediating the binding and internalization of recombinant PrP^{C} (Gauczynski *et al.*, 2001b; Hundt *et al.*, 2001). We identified interaction domains of the cellular prion protein with LRP and proposed a model for the interaction complex of PrP with LRP/LR. In this model, heparan sulfate proteoglycans (HSPGs) would act as co-factors/co-receptors for the

binding and internalization process of PrP (Hundt *et al.*, 2001) and may account for the LRP/LR polymorphism.

The polypeptide predicted from the non-integrin laminin receptor cDNA sequence consists of 295 amino acids and the in vitro translation of selectively hybridized mRNA produced a protein with an apparent molecular weight of 37 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rao *et al.*, 1989). However, other isoforms of the laminin receptor, displaying higher molecular weights, have also been described (Buto *et al.*, 1998; Castronovo *et al.*, 1991; Landowski *et al.*, 1995; Menard *et al.*, 1997). One of these isoforms, the 67 kDa high affinity laminin receptor (LR: presumably the mature form of the receptor), is thought to arise from the heterodimerization of its precursor molecule, the 37 kDa LRP, with a still unidentified molecule (Buto *et al.*, 1998). The 67 kDa LR is believed to be the functional isoform of the receptor regarding its ability to mediate stong attachement of cells to laminin (Lesot *et al.*, 1983; Malinoff and Wicha, 1983; Rao *et al.*, 1983) to be overexpressed on cancer cell suface, and to promote the invasive and metastatic capacity of these cells (Menard *et al.*, 1997). Thus, it is of crucial importance to investigate whether this isoform is normally expressed in mouse brain and whether this isoform binds PrP.

We prepared brain protein fractions from uninfected mouse brain homogenates (abbreviated here as AS50: 50% ammonium sulfate fraction) according to Martins *et al.* (Martins *et al.*, 1997). Using antibodies recognizing specifically the LRP (W3) and the LR (ab711), we questioned what isoforms of the laminin receptor could be evidenced in the AS50 brain fraction. With these polyclonal antibodies, we detected 4 different isoforms of the receptor, migrating at 44 kDa (Figure 1, lane 2), 60 and 67 kDa (Figure 1, lane 1), and 220 kDa (Figure 1, lane 1). The 44 kDa isoform, which has been observed previously in cellular extract such as A431 human epidermoid carcinoma cells, corresponds to the precursor receptor LRP (Buto *et al.*, 1998; Rao *et al.*, 1989). The 60 and 67 kDa doublet bands have also been detected in A431 cellular extract and other cancer tissues (Buto *et al.*, 1998; Castronovo, 1993). The 60 kDa was described as a differentially phosphorylated isoform of the mature 67 kDa Laminin receptor (Buto *et al.*, 1998). The last isoform, the 220 kDa protein, presumably corresponds to an oligomeric form of the LR. The specificity of these antibodies was confirmed since no bands were detected using the secondary antibody alone (Figure 1, lane 3). It is interesting to note the different spectrum of recognition of the laminin receptor with the two antibodies used. In the first case, Ab711, a polyclonal antibody directed against amino acids 263-282 of

the C-terminal domain of human laminin receptor (Wewer *et al.*, 1987) recognizes only the higher molecular weight isoforms of the receptor while the antibody W3 raised against full length LRP (Rieger *et al.*, 1997) recognizes only LRP. This suggests that the corresponding epitopes are exposed differentially whether the receptor is in a precursor or mature state, and heterodimerized or oligomerized.

We next wanted to identify which laminin receptor isoform could interact with PrP. Thus, we performed overlay assays according to Martins *et al.* using human GST-PrP fusion protein (GST-PrP) and the AS50 brain fraction. The integrity of the recombinant protein was first verified by western blot (Figure 2a, lane 1). For the overlay, the proteins present in the AS50 fraction were separated by gel electrophoresis and overlaid with recombinant GST::PrP. Then, using a polyclonal antibody directed against GST, we showed the binding of GST::PrP to several proteins displaying molecular weights of approximately 44 kDa and 60/67 kDa (Figure 2b, lane 1, arrows). The molecular weight of the bands detected corresponded exactly to those detected with LRP/LR antibodies (compare with Figure 1). A mock overlay reveals no unspecific binding of the anti-GST antibody to the AS50 brain fraction (Figure 2b, lane 2). In order to strengthen the demonstration that PrP binds to proteins exhibiting characteristic molecular weights for the different LR isoforms in SDS-PAGE (Figure 1), we repeated the overlay assay using ^{35}S radiolabeled GST::PrP which gives a better resolution of the signal. Validating our previous results, the ^{35}S labeled GST::PrP bound to several proteins migrating in the gel at 60 kDa, 67 kDa, 220 kDa, and weakly to the 44 kDa protein (Figure 2c, lane 1) again demonstrating the interaction between PrP and the laminin receptor. As a control, the specific interaction of GST::PrP to the proteins of the AS50 fraction was verified using ^{35}S radiolabeled GST (Figure 2c, lane 2).

In this study, we identified the isoforms of the high affinity laminin receptor which are expressed in the murine central nervous system and showed that all these isoforms interact with PrP. We demonstrated the specific binding of both non-radiolabeled and radiolabeled GST::PrP to the 44 kDa, 60/67 kDa, and 220 kDa isoforms. The 60/67 kDa isoform, referred to as the mature isoform, is considered to be the functional entity. Therefore, these results are suggestive of an effective role of the PrP/67 kDa LR interaction in the metabolism of PrP^C and presumably its pathological counterpart PrPres. Hence, further investigations of the laminin receptor as a potential therapeutic target for TSE pathologies have to be considered. Moreover, a parallel can be established between our present demonstration and a previously

published study demonstrating the binding of PrP to a 60/66 kDa protein found in the AS50 murine brain fraction (Martins *et al.*, 1997). In this study, Martin's *et al.* exploited a concept called complementary hydropathy, by which peptides encoded by complementary DNA strands bind to each other, and can be used to produce peptides that mimic the binding site of a receptor. Surprisingly, both receptor candidates, i.e. the laminin receptor and the protein isolated by complementary hydropathy (Martins *et al.*, 1997) are found in the 50% ammonium sulfate brain extract, share the same electrophoresis pattern with a doublet band at 60/67 kDa, and exhibit the same PrP binding properties in overlay assays. The 66 kD band of the protein isolated by complementary hydropathy was recently identified as the murine stress-inducible protein 1 (Zanata *et al.*, 2002)

Our study confirms that the non-integrin 67 kDa laminin receptor is present in murine brain and that it binds PrP. This fact along with our previous study (Gauczynski *et al.*, 2001b) demonstrating that the laminin receptor acts as the cell surface receptor internalizing PrP supports the crucial role of this receptor as the cell surface receptor for the prion protein in the brain. In order to better comprehend the pathogenesis of prion diseases and to allow new approaches in therapeutics, the physiological role of the interaction of PrP with the various isoforms of the receptor (44kDa, 60/67 kDa and 220 kDa) will have to be decrypted by further biochemical and cell biological studies.

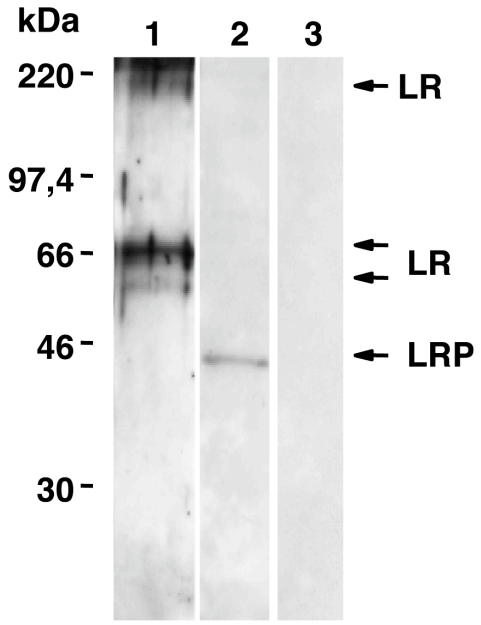


Fig. 1 Antibodies directed against the non-integrin laminin receptor recognize proteins with molecular weight of 44 kDa, 60 kDa, 66 kDa, and 220 kDa.

A mouse brain fraction partially purified by ammonium sulfate precipitation (20 µg) was electrophoretically separated and analyzed by Western blotting using two polyclonal antibodies recognizing either the 37 kDa laminin receptor precursor or the mature 67 kDa laminin receptor. The following polyclonal antibodies were employed in this study: ab711, directed against the peptide P20A (PTEDWSAQPATEDWSAAPTA) amino acids 263-282 from the C-terminal domain of human laminin receptor cDNA, and W3, raised against full length LRP protein.

Methods: Purification of murine laminin receptor by successive ammonium sulfate precipitations. A 20% murine brain homogenate was prepared as previously described (Martins *et al.*, 1997). Briefly, mouse brains were homogenized in a 20% ratio in 50 mM Tris-HCl pH 7. 4, 0.2 % sodium deoxycholate, 0.5% Triton X-100, 1 mM aproptinin, 1 mM leupeptin, 1 mM PMSF, and 1 mM benzamidine and then centrifuged at 12 000g for 30 min. The supernatant was then submitted to successive precipitations with 30% and 50% ammonium sulfate salt. The 50% fraction precipitate (AS50), was then dissolved into 20 mM Tris-HCL pH 7. 4, and 120 mM NaCl. Protein samples were separated on 12% SDS-PAGE gels and transferred to nitrocellulose. The nitrocellulose blots were then blocked in Blotto (Phosphate Buffered Saline (PBS) containing 5% dry skim milk powder and 0.1% Tween 20) for 1 hr and then rinsed with PBS-Tween. Blots were then exposed for 1 hr to anti-laminin receptor polyclonal antibodies ab711 (Abcam ltd, UK) and W3 (Rieger *et al.*, 1997). The blots were washed three times and then exposed to anti-rabbit peroxidase-coupled secondary antibodies (Southern Biotechnology: 1/10000). Peroxidase reactions were detected using enhanced chemiluminescence according to protocols provided by the manufacturer (Amersham).

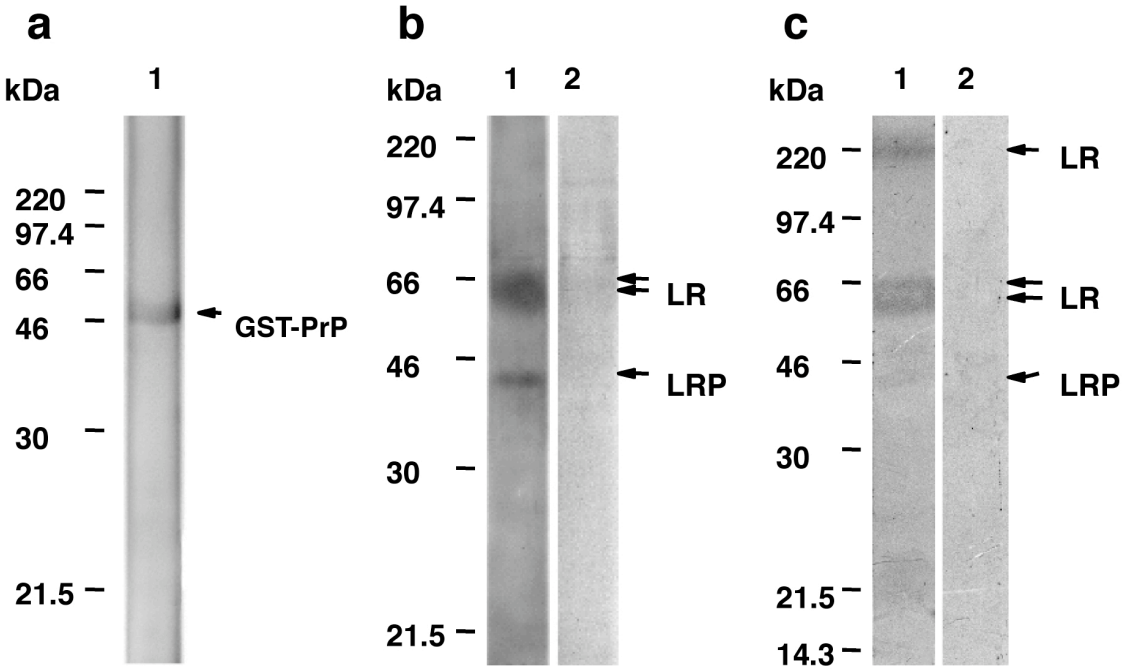


Fig. 2 The proteins recognized by anti-LRP antibodies in the AS50 brain fraction bind PrP. **A**, Verification of the integrity of the recombinant GST::PrP band probed with the PrP-specific antibody 3F4 (lane1). **B**, Proteins from the AS50 brain extract were separated by Western blot and overlaid either with recombinant GST::PrP (lane 1) or with control solution (lane 2). The binding of the GST::PrP was visualized with a polyclonal antibody directed against GST. **C**, Overlay of ^{35}S radiolabeled GST::PrP (lane 1) and GST (lane 2) on immobilized proteins of the AS50 brain fraction.

Methods: All overlay assays were performed on nitrocellulose blots with proteins separated by SDS-PAGE. Blots were incubated with 4 µg/ml of recombinant GST::PrP in PBS containing 0.05% Tween 20 for 3 hrs at room temperature. After three washes in PBS-Tween (0.05%), the immunoblots were further incubated with an anti-GST polyclonal antibody for 1 hr. The reactive bands were visualized with anti-rabbit peroxidase-coupled antibodies. Immunoblots were developed by enhanced chemiluminescence. Overlay assays accomplished with ^{35}S radiolabeled proteins were performed using the same methodology without antibodies, and revealed by exposing X-ray films to the blots.

Purification and expression of heterologous proteins (GST::PrP and GST): the recombinant GST::huPrP23-230 was synthesized in Sf9 cells by infection with the recombinant baculovirus AcSG2T::huPrP23-230 and radiolabeled in the presence of ^{35}S-methionine as described for the GST::haPrP23-231 (Weiss *et al.*, 1995). Radiolabeling of GST was done as for GST::huPrP23-230. Both proteins were purified to homogeneity as described (Weiss *et al.*, 1995).

CHAPTER VII

HIGH-LEVEL EXPRESSION AND CHARACTERISATION OF A GLYCOSYLATED COVALENTLY LINKED DIMER OF THE PRION PROTEIN

published as:

M. Louise Riley, Christoph Leucht, Sabine Gauczynski, Christoph Hundt, Martina Brecelj, Guy Dodson and Stefan Weiss. (2002)
High-level expression and characterisation of a glycosylated covalently linked dimer of the prion protein. *Protein Eng.* **15**, 529-536

Abstract

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

Introduction

Transmissible spongiform encephalopathies (for review: (Lasmézas and Weiss, 2000; Prusiner *et al.*, 1998; Weissmann and Aguzzi, 1997)) are fatal neurodegenerative disorders such as Creutzfeldt-Jakob disease in humans (Creutzfeldt, 1920), bovine spongiform encephalopathy in cattle (Hope *et al.*, 1988) and scrapie in sheep or goat (for review: (Dickinson, 1976)). They are associated with the accumulation of an abnormal form of the prion protein, PrP^{Sc}, derived from the normal cell surface glycoprotein PrP^{c} (Prusiner, 1982). PrP^{c} requires the 37kDa/67 kDa laminin receptor for internalization (Gauczynski *et al.*, 2001b), a process which is thought to require heparan sulfate proteoglycans (HSPGs) mediating the binding of PrP^{c} to its receptor via indirect binding domains (Hundt *et al.*, 2001). The conversion of PrP^{c} to PrP^{Sc} is thought to take place in compartments of the endocytic pathway such as endosomes, lysosomes or endolysosomes (for review see (Gauczynski *et al.*, 2001a)). PrP^{Sc} and PrP^{c} have very different biochemical properties. PrP^{c} is mainly α-helical and is readily degradable by proteinase K, whereas PrP^{Sc} is characterized by an increase in β-sheet conformation, a higher tendency to aggregate, insolubility and proteinase K resistance

(Meyer *et al.*, 1986; Pan *et al.*, 1993; Prusiner *et al.*, 1984). In cases where the disease is transmitted, prion replication appears to involve the interaction between host PrP^{c} and pathogenic PrP^{Sc} from an external source (Prusiner *et al.*, 1984).

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

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

Materials and methods

Reagents and antibodies

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

Plasmid constructions

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

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

Expression in Pichia pastoris

The *P. pastoris* expression system uses the promoter from the alcohol oxidase gene, AOX1, to express heterologous proteins. The expression vector pPICZB (EasySelect Pichia Expression Kit, Invitrogen) was digested with *Eco*RI and *Xba*I and ligated to the inserts. DH5 cells were transformed with the ligation products and plated on low salt LB/zeocin medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, and 25 µg/ml zeocin). The transformants were tested by restriction analysis, and positive clones were amplified to make larger amounts of DNA.

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

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

Larger-scale expression and optimization

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

Expression in the presence of tunicamycin

Tunicamycin was used to block *in vivo* glycosylation. It was added to 10ml cultures of the covalently-linked dimer and monomer (from a stock solution of 1mg/ml in 0.1M NaOH) to a final concentration of 15µg tunicamycin/ml culture. Small-scale expression was carried out essentially as described above, with tunicamycin being included in the BMGY and BMMY culture media. 1ml aliquots were removed 24 hours after induction and expression of the covalently linked dimer and monomer in the cell lysate was analysed by SDS-PAGE and Western blotting. The monoclonal antibody 3B5, which recognises the octarepeat region of human and bovine PrP, was used for protein detection.

Cell lysis and sensitivity to proteinase K

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

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

samples were analysed by immunoblotting (with the 3B5 *and 3F4 antibody*) after of SDS-PAGE.

Sensitivity to endoglycosidase H

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

Purification of yeast plasma membrane fraction

The plasma membrane fractions of yeast overexpressing the FLAG fusion proteins were purified using standard procedures (Panaretou and Piper, 1996). *Pichia pastoris* culture pellets (from 50ml cultures) were resuspended in 10ml cold lysis buffer (25mM imidazole, pH 7.0, 2mM EDTA, 0.4M sucrose). The cells were re-pelleted by centrifugation and the supernatants discarded. Two ml of glass beads and 2ml of lysis buffer were added and cells were broken by vortexing as described above. 9ml of cold lysis buffer was added and the cell debris and glass beads were pelleted by centrifugation (530g, 20 mins, 4°C). The supernatant was removed and centrifuged (22 000g, 30minutes, 4°C) to pellet the plasma and mitochondria fractions. The supernant (cytosolic fraction) was removed and the pellet taken up in TBS containing 5% Triton X-100. This was further diluted to 20ml with TBS containing 0.1% sarcosine, 0.1% NP-40 and 100mM dithiothreitol.

Immunoprecipitation

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

Removal of GPI anchor by cleavage with enterokinase

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

SDS – polyacrylamide gel electrophoresis and immunoblotting

Protein samples were separated on 12% Mighty Small gels according to the manufacturer's protocol (Hoefer, Pharmaciea Biotech Inc. San Francisco, CA) and transferred electrophoretically onto pre-wetted polyvinyldifluoride membranes. The blots were incubated with an anti-PrP antibody (3F4, 3B5, 1:5000 dilution) or with an anti-FLAG M2 antibody (1:600 dilution). The incubation steps were preformed as described previously (Weiss *et al.*, 1995; Weiss *et al.*, 1996) and the bound antibody was visualized with 3,3'-diaminobenzidine tetrahydrochloride.

Results

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

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

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

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

Effect of tunicamycin and endoglycosidase H sensitivity

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

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

there is some residual glycosylation which may be consistent with the covalent prion dimer having some tertiary structure.

Proteinase K sensitivity

In order to analyse the resistance of the covalently linked dimer to proteinase K (PK) and to compare it with huPrP::FLAG expressed in the same system, the cell lysate supernatants were incubated with 0, 2 and 4 µg/ml PK for 1 hour at 37°C. Analysis by SDS-PAGE and Western blotting *employing the 3B5 antibody* (Figure 5) *and the 3F4 antibody (data not shown)* showed that the fusion proteins have similar PK sensitivity, both being completely digested by 4µg/ml PK. Evidently PK is able to degrade the prion monomer and covalent dimer equivalently.

Secretion of the fusion proteins to the plasma membrane

The plasma membrane fractions of *Pichia pastoris* overexpressing huPrP::FLAG and PrP::FLAG::PrP were isolated and analysed by Western blotting (Figure 6B and C). Both fusion proteins were detected in the plasma membrane fraction *and in the cytosolic fraction of the cells.* Coomassie blue staining confirms that the covalently linked dimer is overexpressed and *transported* to the cell membrane (Figure 6A, Lane 1). *This finding is in good harmony with the fact that both of our proteins are glycosylated.*

Immunoprecipitation

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

Enterokinase cleavage

Enterokinase is a highly specific serine protease which cleaves after the carboxy-terminal lysine of the recognition sequence Asp-Asp-Asp-Asp-Lys. This is the last five amino acids of the FLAG –tag. Enterokinase was used to remove the final lysine of the FLAG peptide and the GPI anchor of huPrP::FLAG. The expressed dimer was also treated with enterokinase even though it has two potential cleavage sites.

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

Discussion

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

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

The physical state of the recombinant prion protein, monomer or covalent dimer, is unclear at present. The endoglycosidase H studies suggest that the covalently linked PrP dimer has some three-dimensional structure, stable enough to interfere with the deglycosylation by the

enzyme. Equally the effects of tunicamycin in abolishing glycosylation are less complete with the covalent dimer. The proteinase K sensitivity status of the FLAG tagged prion protein and the covalently linked PrP dimer, however, proved to be similar. This suggests neither recombinant protein has the PrP^{Sc} structure, which is PK resistant (Taraboulos *et al.*, 1990). The similarity in their cleavage properties is however not inconsistent with the covalent dimer retaining some tertiary structure. We suppose that the structure of the covalently linked PrP dimer reported here might be different from the structure of the crystallized PrP dimer where the N- and C-termini of the two chains appear to be very far apart (Knaus *et al.*, 2001). The organization of our covalently linked dimer might also be different from other PrP dimers observed.

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

We proved that our recombinant FLAG tagged prion protein expressed in Pichia pastoris is highly glycosylated and differs in this respect from other non-glycosylated bacterially expressed prion proteins (Riek *et al.*, 1996; Riek *et al.*, 1997). Further structural studies with our glycosylated prion protein will prove whether glycosylations will influence the secondary/tertiary structure of the prion protein.

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

infected or scrapie infected neuroblastoma cells, investigating again its role in the PrP^{c} and PrP^{Sc} propagation process.

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

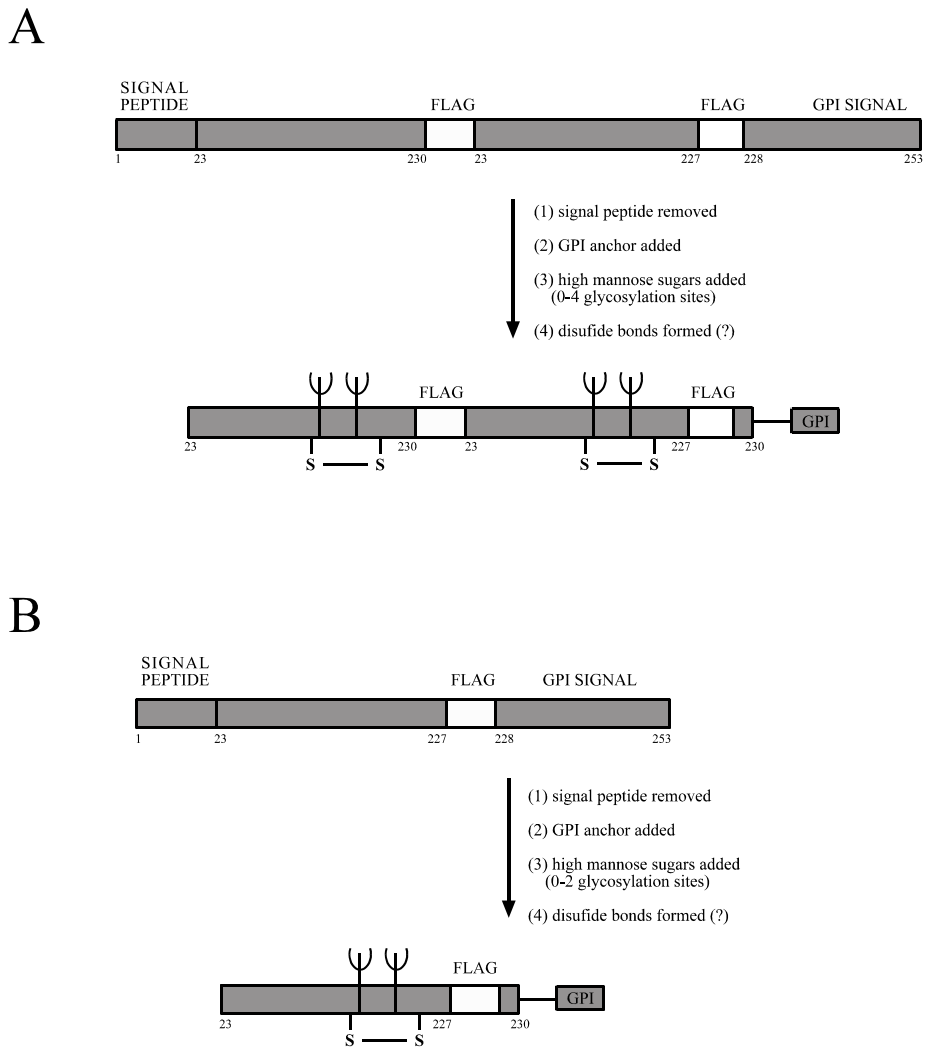


Fig. 1 Schematic diagram of FLAG-tagged PrP constructs and processing in the yeast cell. Both amino- and carboxyl terminal fragments are removed. The GPI anchor and high mannose glycans are added and the proteins are secreted to the cell surface. **(A)** Human PrP-covalently linked to another huPrP via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

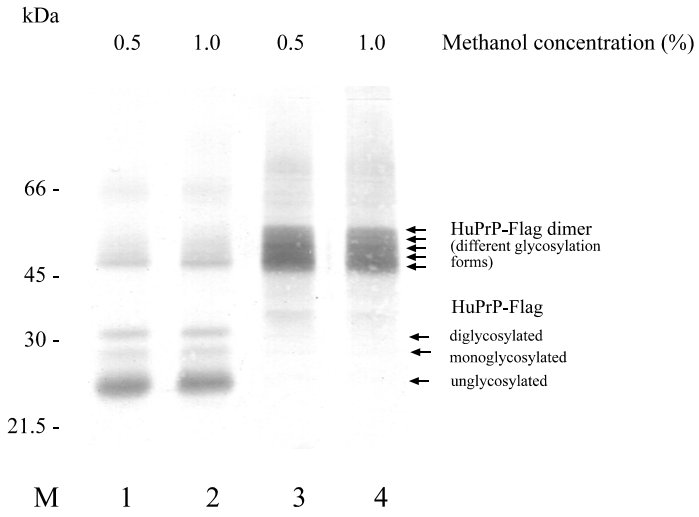


Fig. 2 Expression of FLAG-fusion proteins monitored by SDS-PAGE and Western blot analysis. Shown is a 12% polyacrylamide gel, immunodetection was carried out with the 3B5 antibody. (Lane 1) Lysate of cells expressing PrP::FLAG in 0.5% methanol; (Lane 2) PrP::FLAG in 1.0% methanol; (Lane 3) PrP::FLAG::PrP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

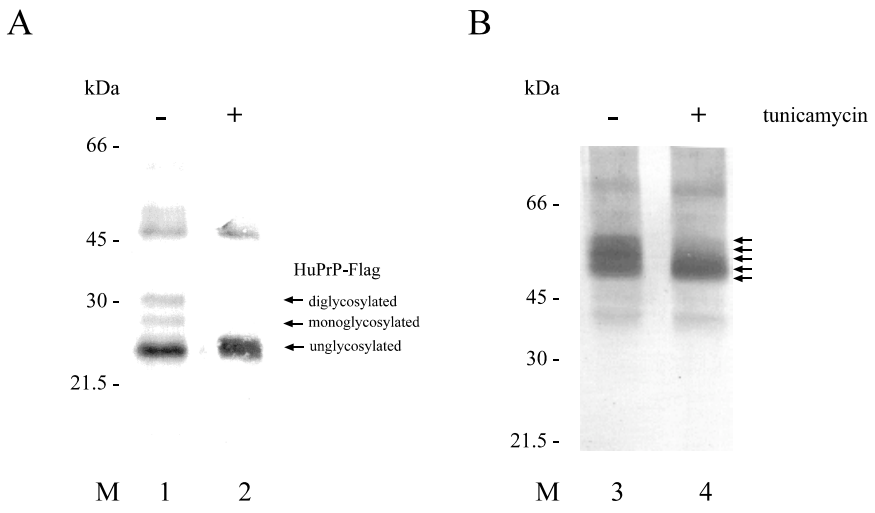


Fig. 3. Expression of FLAG-fusion proteins in the presence or absence of tunicamycin monitored by SDS-PAGE and Western blot analysis, immunodetection was carried out with the 3B5 antibody. (Lane 1) Lysate of cells expressing PrP::FLAG in the absence and (Lane 2) in the presence of 15µg/ml tunicamycin.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

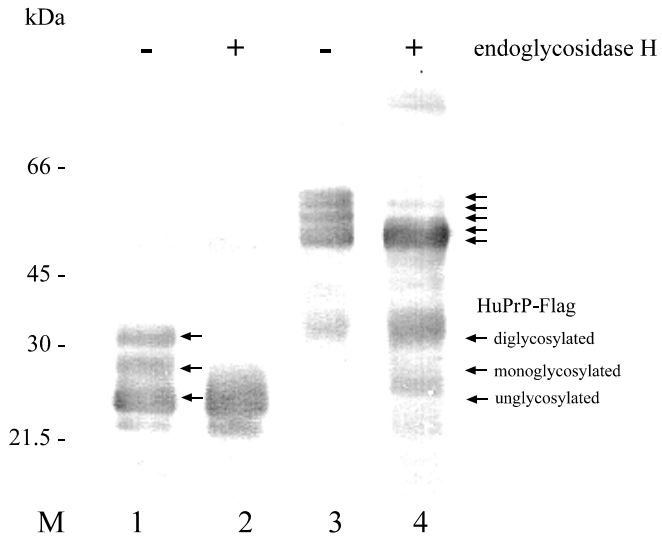


Fig. 4 Digestion with endoglycosidase H, monitored by SDS-PAGE and Western blot analysis. Immunodetection was performed with the 3B5 antibody. (Lanes1-2) Lysate supernatants of cells expressing PrP::FLAG, treated with 0 (Lane1) and 0.5 (Lane 2) units/ml endoglycosidase H. (Lanes 3-4) Lysate supernatants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

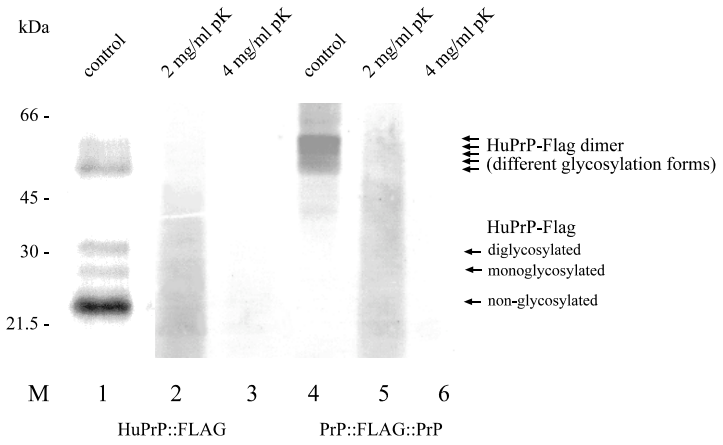


Fig. 5 Digestion with proteinase K, monitored by SDS-PAGE and Western blot analysis. Immunodetection is with the 3B5 antibody. (Lanes1-3) Lysates of cells expressing PrP::FLAG, digested with 0 (Lane 1), 2 (Lane 2) and 4 (Lane 3) µg/ml proteinase K. (Lanes 4-6) Lysates of cells expressing PrP::FLAG::PrP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

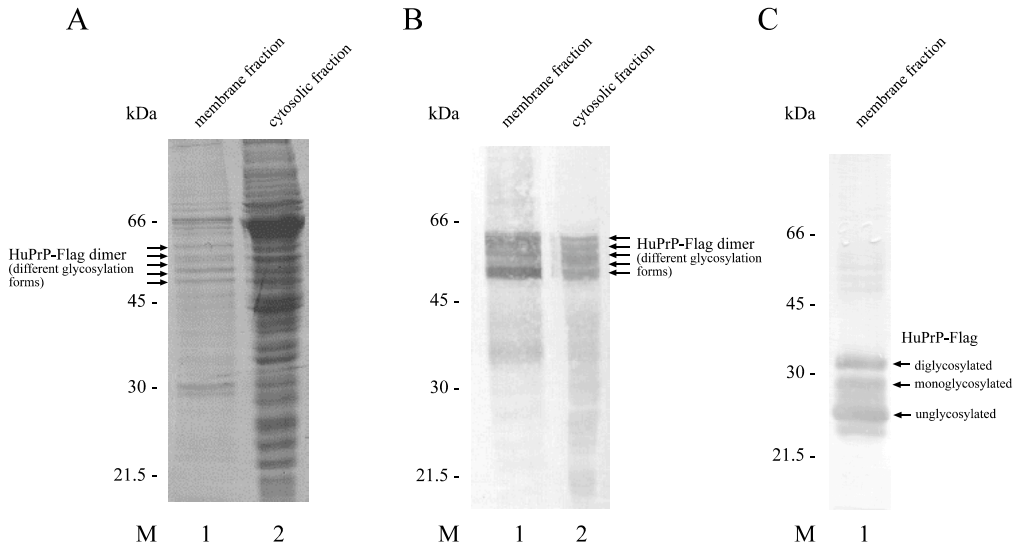


Fig. 6 Isolation of crude plasma membrane fractions **(A)** from yeast cells overexpressing PrP:FLAG::PrP, analysed by SDS-PAGE and Coomassie blue staining. (Lane 1) membrane fraction (Lane 2) cytosolic fraction, **(B)** analysed by Western blotting using the 3B5 antibody. (Lane 1) membrane fraction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

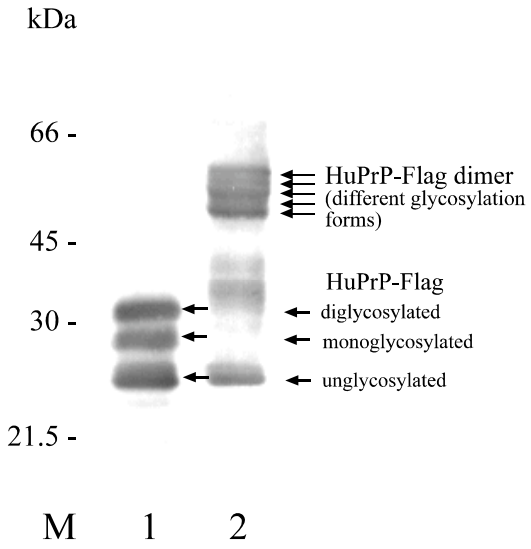


Fig.7 Immunoprecipitation of FLAG-fusion proteins monitored by Western blotting, with the 3B5 antibody. (Lane 1) huPrP::FLAG (Lane 2) PrP::FLAG::PrP.

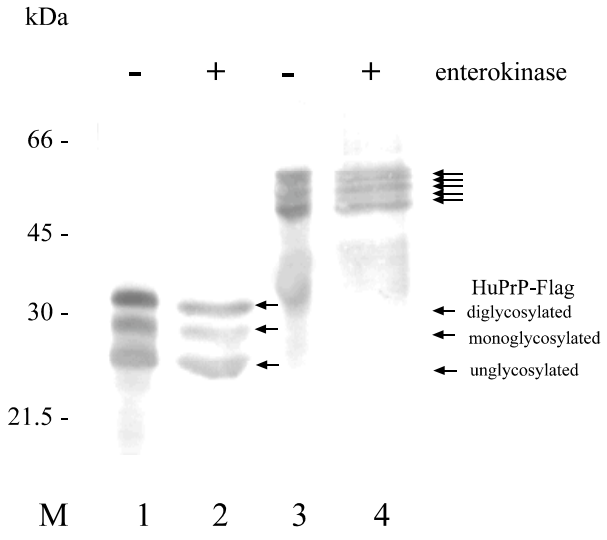


Fig. 8 Enterokinase cleavage of FLAG-fusion proteins monitored by Western Blotting employing the 3B5 antibody. (Lane 1) untreated huPrP::FLAG, (Lane 2) enterokinase treated huPrP::FLAG, (Lane 3) untreated PrP::FLAG::PrP, (Lane 4) enterokinase treated PrP::FLAG::PrP.

CHAPTER VIII

REFERENCES

Aguzzi, A. and Weissmann, C. (1998) Prion diseases. *Haemophilia*, **4**, 619-627.

Albelda, S.M. and Buck, C.A. (1990) Integrins and other cell adhesion molecules. *FASEB J.*, **4**, 2868-2880.

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

Alper, T., Haig, D.A. and Clarke, M.C. (1966) The exceptionally small size of the scrapie agent. *Biochem. Biophys. Res. Commun.*, **22**, 278-284.

Anderson, R.G. (1993) Plasmalemmal caveolae and GPI-anchored membrane proteins. *Curr Opin Cell Biol*, **5**, 647-652.

Ardini, E., Pesole, G., Tagliabue, E., Magnifico, A., Castronovo, V., Sobel, M.E., Colnaghi, M.I. and Menard, S. (1998) The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol. Biol. Evol.*, **15**, 1017-1025.

Asante, E.A., Linehan, J.M., Desbruslais, M., Joiner, S., Gowland, I., Wood, A.L., Welch, J., Hill, A.F., Lloyd, S.E., Wadsworth, J.D. and Collinge, J. (2002) BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J*, **21**, 6358-6366.

Askanas, V., Bilak, M., Engel, W.K., Leclerc, A. and Tome, F. (1993) Prion protein is strongly immunolocalized at the postsynaptic domain of human normal neuromuscular junctions. *Neurosci Lett*, **159**, 111-114.

Auth, D. and Brawerman, G. (1992) A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. *Proc. Natl Acad. Sci. U S A*, **89**, 4368-4372.

Bao, Q. and Hughes, R.C. (1995) Galectin-3 expression and effects on cyst enlargement and tubulogenesis in kidney epithelial MDCK cells cultured in three-dimensional matrices in vitro. *J Cell Sci*, **108**, 2791-2800.

Basler, K., Oesch, B., Scott, M., Westaway, D., Wälchli, M., Groth, D.F., McKinley, M.P., Prusiner, S.B. and Weissmann, C. (1986) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell*, **46**, 417-428.

Bateman, D., Hilton, D., Love, S., Zeidler, M., Beck, J. and Collinge, J. (1995) Sporadic Creutzfeldt-Jakob disease in a 18-year-old in the UK. *Lancet*, **346**, 1155-1156.

Bazan, J.F., Fletterick, R.J., McKinley, M.P. and Prusiner, S.B. (1987) Predicted secondary structure and membrane topology of the scrapie prion protein. *Protein Eng*, **1**, 125-135.

Beck, K., Hunter, I. and Engel, J. (1990) Structure and function of laminin: anatomy of a multidomain glycoprotein. *FASEB J.*, **4**, 148-160.

Bessen, R.A., Kocisko, D.A., Raymond, G.J., Nandan, S., Lansbury, P.T. and Caughey, B. (1995) Non-genetic propagation of strain-specific properties of scrapie prion protein. *Nature*, **375**, 698-700.

Bosque, P.J. and Prusiner, S.B. (2000) Cultured cell sublines highly susceptible to prion infection. *Journal of virology*, **74**, 4377-4386.

Bounhar, Y., Zhang, Y., Goodyer, C.G. and LeBlanc, A.C. (2001) Prion protein protects human neurons against Bax-mediated apoptosis. *J. Biol. Chem.*, **24**, [epub ahead of print].

Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C. and Aguzzi, A. (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*, **379**, 339-343.

Brimacombe, D.B., Bennett, A.D., Wustemann, F.S., Gill, A.C., Damm, J.C. and Bostock, C.J. (1999) Characterisation and polyanion-binding properties of purified recombinant prion protein. *Biochem. J.*, **342**, 605-613.

Britton, T.C., al Sarraj, S., Shaw, C., Campbell, T. and Collinge, J. (1995) Sporadic Creutzfeldt-Jakob disease in a 16-year-old in the UK. *Lancet*, **346**, 1155.

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

Brown, D.R., Wong, B.-S., Hafiz, F., Clive, C., Haswell, S.J. and Jones, I.M. (1999) Normal prion protein has an activity like that of superoxide dismutase. *Biochem. J.*, **344**, 1-5.

Brown, P., Goldfarb, L., Brown, W., Goldgaber, D., Rubenstein, R., Kascsak, R., Guiroy, D., Piccardo, P., Boellaard, J. and Gajdusek, D. (1991a) Clinical and molecular genetic study of a large German kindred with Gerstmann-Straussler-Scheinker syndrome. *Neurology*, **41**, 375-379.

Brown, P., Goldfarb, L.G., Gibbs, C.J.J. and Gajdusek, D.C. (1991b) The phenotypic expression of different mutations in transmissible familial Creutzfeldt-Jakob disease. *Eur. J. Epidemiol.*, **7**, 469-476.

Brown, P., Goldfarb, L.G., Kovanen, J., Haltia, M., Cathala, F., Sulima, M., Gibbs, C.J.J. and Gajdusek, D.C. (1992) Phenotypic characteristics of familial Creutzfeldt-Jakob disease associated with the codon 178Asn PRNP mutation. *Ann. Neurol.*, **31**, 282-285.

Brown, P., Preece, M., Brande, l.J.P., Sato, T., McShane, L., Zerr, I., Fletcher, A., Will, R.G., Pocchiari, M., Cashman, N.R., d'Aignaux, J.H., Cervenakova, L., Fradkin, J., Schonberger, L.B. and Collins, S.J. (2000) Iatrogenic Creutzfeldt-Jakob disease at the millennium. *Neurology 2000 Oct 24;55(8):1075-81*, **55**, 1075-1081.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H. and Bostock, C.J. (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **388**, 498-501.

Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. *Cell*, **73**, 1339-1347.

Bueler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H.P., DeArmond, S.J., Prusiner, S.B., Aguet, M. and Weissmann, C. (1992) Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature*, **356**, 577-582.

Burns, C.S., Aronoff Spencer, E., Dunham, C.M., Lario, P., Avdievich, N.I., Antholine, W.E., Olmstead, M.M., Vrielink, A., Gerfen, G.J., Peisach, J., Scott, W.G. and Millhauser, G.L. (2002) Molecular features of the copper binding sites in the octarepeat domain of the prion protein. *Biochemistry*, **41**, 3991-4001.

Buto, S., Tagliabue, E., Ardini, E., Magnifico, A., Ghirelli, C., van den Brule, F., Castronovo, V., Colnaghi, M.I., Sobel, M.E. and Menard, S. (1998) Formation of the 67-kDa laminin receptor by acylation of the precursor. *J. Cell. Biochem.*, **69**, 244-251.

Callahan, M.A., Xiong Lw, L. and Caughey, B. (2001) Reversibility of scrapie-associated prion protein aggregation. *J Biol. Chem.*, **25**, 25.

Canfield, S.M. and Khakoo, A.Y. (1999) The nonintegrin 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 avid cellular adherence to laminin. *J. Immunol.*, **163**, 3430-3440.

Castronovo, V. (1993) Laminin receptors and laminin-binding proteins during tumor invasion and metastasis. *Invasion Metastasis*, **13**, 1-30.

Castronovo, V., Claysmith, A.P., Barker, K.T., Cioce, V., Krutzsch, H.C. and Sobel, M.E. (1991a) Biosynthesis of the 67 kDa high affinity laminin receptor. *Biochem. Biophys. Res. Commun.*, **177**, 177-183.

Castronovo, V., Taraboletti, G. and Sobel, M.E. (1991b) Functional domains of the 67-kDa laminin receptor precursor. *J. Biol. Chem.*, **266**, 20440-20446.

Caughey, B., Brown, K., Raymond, G.J., Katzenstein, G.E. and Thresher, W. (1994) Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red [corrected] [published erratum appears in J. Virol. 1994 Jun;68(6):4107]. *J. Virol.*, **68**, 2135-2141.

Caughey, B., Horiuchi, M., Demaimay, R. and Raymond, G.J. (1999) Assays of protease-resistant prion protein and its formation. *Methods Enzymol*, **309**, 122-133.

Caughey, B. and Raymond, G.J. (1991) The scrapie-associated form of PrP is made from a cell surface precursor that is both protease and phospholipase-sensitive. *J. Biol. Chem.*, **266**, 18217-18223.

Caughey, B., Raymond, G.J., Ernst, D. and Race, R.E. (1991a) N-terminal truncation of the Scrapie-associated form of PrP by lysosomal protease(s): Implications regarding the site of conversion of PrP to the protease-resistant state. *J. Virol.*, **65**, 6597-6603.

Caughey, B.W., Dong, A., Bhat, K.S., Ernst, D., Hayes, S.F. and Winslow, S.C. (1991b) Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy . *Biochemistry*, **30**, 7672-7680.

Cervenakova, L., Buetefisch, C., Lee, H., Taller, I., Stone, G., Gibbs, C.J., P, B., Hallett, M. and Goldfarb, L.G. (1999) Novel PRNP sequence variant associated with familial encephalopathy. *Am. J. Med. Genet.*, **88**, 653-656.

Cervenakova, L., Goldfarb, L.G., Garruto, R., Lee, H.S., Gajdusek, D.C. and Brown, P. (1998) Phenotype-genotype studies in kuru: Implications for new variant Creutzfeldt-Jakob disease. *Proc. Natl. Acad. Sci. USA,* **95**, 13239-13241

Chandler, R.L. (1961) Encephalopathy in mice by inoculation of scrapie brain material. *Lancet*, **I**, 1378-1379.

Chen, S.G., Teplow, D.B., Parchi, P., Teller, J.K., Gambetti, P. and Autilio, G.L. (1995) Truncated forms of the human prion protein in normal brain and in prion diseases. *J. Biol. Chem.*, **270**, 19173-19180.

Chesebro, B., Race, R., Wehrly, K., Nishio, J., Bloom, M., Lechner, D., Bergstrom, S., Robbins, K., Mayer, L., Keith, J. and al., e. (1985) Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. *Nature*, **315**, 331-333.

Christophe, T., Karlsson, A., Dugave, C., Rabiet, M.J., Boulay, F. and Dahlgren, C. (2001) The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/LXA4R and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J. Biol. Chem.*, **2**, 2.

Collinge, J. and Palmer, M.S. (1992) Molecular genetics of inherited, sporadic and iatrogenic prion disease. In Prusiner, S.B., Collinge, J., Powell, J. and Anderton, B. (eds.), *Prion diseases of humans and animals*. Ellis Horwood Limited, Chichester, pp. 95-119.

Collinge, J., Sidle, K.C., Meads, J., Ironside, J. and Hill, A.F. (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD [see comments]. *Nature*, **383**, 685-690.

Collinge, J., Whittington, M.A., Sidle, K.C.L., Smith, C.J., Palmer, M.S., Clarke, A.R. and Jefferys, J.G.R. (1994) Prion protein is necessary for normal synaptic function. *Nature*, **370**, 295-297.

Collins, S., Law, M.G., Fletcher, A., Boyd, A., Kaldo, r.J. and Masters, C.L. (1999) Surgical treatment and risk of sporadic Creutzfeldt-Jakob disease: a case-control study. *Lancet*, **353**, 693-697.

Creutzfeldt, H.G. (1920) Über eine eigenartige Erkrankung des Zentralnervensystems. Vorläufige Mitteilung. *Z. f. d. ges. Neurol. und Psych.*, 1-18.

Cuille, J. and Chelle, P.L. (1939) Experimantal transmission of trembling to the goat. *Seances Acad. Sci.*, **208**, 1058-1160.

Davis, S.C., Tzagoloff, A. and Ellis, S.R. (1992) Characterization of a yeast mitochondrial ribosomal protein structurally related to the mammalian 68-kDa high affinity laminin receptor. *J. Biol. Chem.*, **267**, 5508-5514.

Dickinson, A.G. (1976) Scrapie in sheep and goats. *Front Biol*, **44**, 209-241.

Doh-ura, K., Tateishi, J., Sasaki, H., Kitamoto, T. and Sakaki, Y. (1989) Pro-leu change at position 102 of prion protein is the most common but not the sole mutation related to Gerstmann-Straussler syndrome. *Biochem. Biophys. Res. Commun.*, **163**, 974-979.

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

Douville, P.J. and Carbonetto, S. (1992) Genetic linkage analysis in recombinant inbred mice of P40, a putative clone for the high-affinity laminin receptor. *Mamm. Genome*, **3**, 438-446.

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

El Nemer, W., Gane, P., Colin, Y., Bony, V., Rahuel, C., Galacteros, F., Cartron, J.P. and Le Van Kim, C. (1998) The Lutheran blood group glycoproteins, the erythroid receptors for laminin, are adhesion molecules. *J. Biol. Chem.*, **273**, 16686-16693.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 2001 May 24;411(6836):494-8*, **411**, 494-498.

Enright, A.J., Van Dongen, S. and Ouzounis, C.A. (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res.*, **30**, 1575-1584.

Erickson, R.P., Lai, L.W. and Grimes, J. (1993) Creating a conditional mutation of Wnt-1 by antisense transgenesis provides evidence that Wnt-1 is not essential for spermatogenesis. *Dev. Genet.*, **14**, 274-281.

Fernandez, M.-T., Castronovo, V., Rao, C.N. and Sobel, M.E. (1991) The high affinity murine laminin receptor is a member of a multicopy gene family. *Biochem. Biophys. Res. Commun.*, **175**, 84-90.

Fournier, J.G., Escaig, H.F., Billette, d.V.T. and Robain, O. (1995) Ultrastructural localization of cellular prion protein (PrPc) in synaptic boutons of normal hamster hippocampus. *C. R. Acad. Sci. III*, **318**, 339-344.

Gabizon, R., Meiner, Z., Halimi, M. and Ben, S.S. (1993) Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. *J. Cell. Physiol.*, **157**, 319-325.

Gabus, C., Derrington, E., Leblanc, P., Chnaiderman, J., Dormont, D., Swietnicki, W., Morillas, M., Surewicz, W.K., Marc, D., Nandi, P. and Darlix, J.L. (2001) The prion protein has RNA binding and chaperoning properties characteristic of nucleocapsid protein NCP7 of HIV-1. *J. Biol. Chem.*, **276**, 19301-19309.

Gajdusek, D.C. (1996) Infectious amyloids: subacute spongiform encephalopathies as transmissible cerebral amyloidoses. In Fields, B.N., Knipe, D.M. and Howley, P.M. (eds.), *Fields virology. 3rd ed.* Lippencott-Raven Publishers, Philadelphia, Pennsylvania, pp. 2851-2899.

Gajdusek, D.C., Gibb, W.R. and Alpers, M. (1966) Experimental Transmission of a Kuru-like Syndrome to Chimpanzees. *Nature*, **209**, 794-796.

Gajdusek, D.C. and Zigas, V. (1957) Degenerative disease of the central nervous system in New Guinea. *New Eng. J. Med.*, **257**, 974-978.

Gajdusek, D.C. and Zigas, V. (1959) Kuru. Clinical, pathological and epidemiological study of an acute progressive degenerative disease of the central nervous system among natives of the Eastern Highland of New Guinea. *Am. J. Med.*, **1959**, 442-469.

Gambetti, P. and Lugaresi, E. (1998) Conclusions of the symposium. B*rain Pathol. 1998 Jul;8(3):571-5*, **8**, 571-575.

Garcia-Hernandez, M., Davies, E. and Staswick, P.E. (1994) Arabidopsis p40 homologue. A novel acidic protein associated with the 40 S subunit of ribosomes. *J. Biol. Chem.*, **269**, 20744-20749.

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

Gauczynski, S., Peyrin, J.M., Haik, S., Leucht, C., Hundt, C., Rieger, R., Krasemann, S., Deslys, J.P., Dormont, D., Lasmezas, C.I. and Weiss, S. (2001b) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J.*, **20**, 5863-5875.

Gerstmann, J., Sträussler, E. and Scheinker, I. (1936) Über eine eigenartige hereditär-familiäre Erkrankung des Zentralnervensystems. Zugleich ein Beitrag zur Frage des vorzeitigen lokalen Alterns. *Z. f. d. ges. Neurol. und Psych.*, **154**, 736-762.

Gibbs, C.J.J., Gajdusek, D.C., Asher, D.M., Alpers, M.P., Beck, E., Daniel, P.M. and Matthews, W.B. (1968) Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science*, **161**, 388-389.

Goldfarb, L., Brown, P., Haltia, M., Cathala, F., McCombie, W.R., Kovanen, J., Cervenakova, L., Goldin, L., Nieto, A., Godec, M.S. and et al., (1992) Creutzfeldt-Jakob disease cosegregates with the codon 178Asn PRNP mutation in families of European origin. *Ann. Neurol.*, **31**, 274-281.

Goldfarb, L.G., Brown, P., Cervenakova, L. and Gajdusek, D.C. (1994) Molecular genetic studies of Creutzfeldt-Jakob disease. *Mol. Neurobiol.*, **8**, 89-97.

Goldfarb, L.G., Korczyn, A.D., Brown, P., Chapman, J. and Gajdusek, D.C. (1990a) Mutation in codon 200 of scrapie amyloid precursor gene linked to Creutzfeldt-Jakob disease in Sephardic Jews of Libyan and non-Libyan origin. *Lancet*, **336**, 637-638.

Goldfarb, L.G., Mitrova, E., Brown, P., Toh, B.K. and Gajdusek, D.C. (1990b) Mutation in codon 200 of scrapie amyloid protein gene in two clusters of Creutzfeldt-Jakob disease in Slovakia. *Lancet*, **336**, 514-515.

Graner, E., Mercadante, A.F., Zanata, S.M., Forlenza, O.V., Cabral, A.L., Veiga, S.S., Juliano, M.A., Roesler, R., Walz, R., Minetti, A., Izquierdo, I., Martins, V.R. and Brentani, R.R. (2000) Cellular prion protein binds laminin and mediates neuritogenesis. *Brain Res. Mol. Brain Res.*, **76**, 85-92.

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

Harris, D.A. (1999) Cell biological studies of the prion protein. In Harris, D.A. (ed.), *Prions: Molecular and Cellular Biology.* Horizon Scientific Press, Norfolk, UK, pp. 53-65.

Harris, D.A., Gorodinsky, A., Lehmann, S., Moulder, K. and Shyng, S.L. (1996) Cell biology of the prion protein. *Curr. Top. Microbiol. Immunol.*, **207**, 77-93.

Hegde, R.S., Mastrianni, J.A., Scott, M.R., DeFea, K.A., Tremblay, P., Torchia, M., DeArmond, S.J., Prusiner, S.B. and Lingappa, V.R. (1998) A transmembrane form of the prion protein in neurodegenerative disease. *Science*, **279**, 827-834.

Heppner, F.L., Musahl, C., Arrighi, I., Klein, M.A., Rulicke, T., Oesch, B., Zinkernagel, R.M., Kalinke, U. and Aguzzi, A. (2001) Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. *Science*, **294**, 178-182.

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

Hinek, A., Wrenn, D.S., Mecham, R.P. and Barondes, S.H. (1988) The elastin receptor: a galactoside binding protein. *Science*, **239**, 1539-1541.

Hope, J., Reekie, L.J., Hunter, N., Multhaup, G., Beyreuther, K., White, H., Scott, A.C., Stack, M.J., Dawson, M. and Wells, G.A. (1988) Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. *Nature*, **336**, 390-392.

Horiuchi, M., Priola, S.A., Chabry, J. and Caughey, B. (2000) Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers. *Proc. Natl. Acad. Sci. USA*, **97**, 5836-5841.

Hsiao, K., Meiner, Z., Kahana, E., Cass, C., Kahana, I., Avrahami, D., Scarlato, G., Abramsky, O., Prusine, r.S.B. and Gabizon, R. (1991) Mutation of the prion protein in Libyan Jews with Creutzfeldt-Jakob disease. *N. Engl. J. Med.*, 1091-1097.

Hsiao, K. and Prusiner, S.B. (1990) Inherited human prion diseases. *Neurology 1990 Dec;40(12):1820-7*, **40**, 1820-1827.

Hundt, C., Gauczynski, S., Leucht, C., Riley, M.-L. and Weiss, S. (accepted) Intra- and interspecies interactions of prion proteins and effects of mutations and polymorphisms. *Biol. Chem.*

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

Ironside, J.W. (1998) Prion diseases in men. *J. Pathol.*, **186**, 227-234.

Jackers, P., Clausse, N., Fernandez, M., Berti, A., Princen, F., Wewer, U., Sobel, M.E. and Castronovo, V. (1996a) Seventeen copies of the human 37 kDa laminin receptor precursor/p40 ribosome-associated protein gene are processed pseudogenes arisen from retropositional events. *Biochim. Biophys. Acta*, **1305**, 98-104.

Jackers, P., Minoletti, F., Belotti, D., Clausse, N., Sozzi, G., Sobel, M.E. and Castronovo, V. (1996b) Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p40 at chromosome 3p21.3. *Oncogene*, **13**, 495-503.

Jackson, G.S., Hosszu, L.L., Power, A., Hill, A.F., Kenney, J., Saibil, H., Craven, C.J., Waltho, J.P., Clarke, A.R. and Collinge, J. (1999) Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations. *Science*, **283**, 1935-1937.

Jackson, G.S., Murray, I., Hosszu, L.L., Gibbs, N., Waltho, J.P., Clarke, A.R. and Collinge, J. (2001) Location and properties of metal-binding sites on the human prion protein. *Proc. Natl Acad. Sci. USA*, **98**, 8531-8535.

Jaegly, A., Mouthon, F., Peyrin, J.M., Camugli, B., Deslys, J.P. and Dormont, D. (1998) Search for a nuclear localization signal in the prion protein. *Molecular and Cellular Neurosciences*.

Jakob, A. (1921) Über eigenartige Erkrankungen des Zentralnervensystems mit bemerkenswerten anatomischen Befunde (spastische Pseudosklerose-Encephalomyelopathie mit disseminierten Degenerationsherden). Vorläufige Mitteilung. *Z. Ges. Neurol. Psychiatr.*, **64**, 147-228.

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

Jarrett, J.T. and Lansbury, P.T., Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell*, **73**, 1055-1058.

Katsuki, M., Sato, M., Kimura, M., Yokoyama, M., Kobayashi, K. and Nomura, T. (1988) Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice. *Science*, **241**, 593-595.

Kazmin, D.A., Hoyt, T.R., Taubner, L., Teintze, M. and Starkey, J.R. (2000) Phage display mapping for peptide 11 sensitive sequences binding to laminin-1. *J. Mol. Biol.*, **298**, 431-445.

Keppel, E. and Schaller, H.C. (1991) A 33 kDa protein with sequence homology to the 'laminin binding protein' is associated with the cytoskeleton in hydra and in mammalian cells. *J. Cell. Science*, **100**, 789-797.

Kinoshita, K., Kaneda, Y., Sato, M., Saeki, Y., Wataya, K.M. and Hoffmann, A. (1998) LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. *Biochem. Biophys. Res. Commun.*, **253**, 277-282.

Kitamoto, T., Shin, R.W., Doh, u.K., Tomokane, N., Miyazono, M., Muramoto, T. and Tateishi, J. (1992) Abnormal isoform of prion proteins accumulates in the synaptic structures of the central nervous system in patients with Creutzfeldt-Jakob disease. *Am. J. Pathol.*, **140**, 1285-1294.

Klatzo, I., Gajdusek, D.C. and Zigas, V. (1959) Pathology of Kuru. *Lab Invest*, **8**, 799-847.

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

Kocisko, D.A., Come, J.H., Priola, S.A., Chesebro, B., Raymond, G.J., Lansbury, P.T. and Caughey, B. (1994) Cell-free formation of protease-resistant prion protein [see comments]. *Nature*, **370**, 471-474.

Krasemann, S., Groschup, M.H., Harmeyer, S., Hunsmann, G. and Bodemer, W. (1996) Generation of monoclonal antibodies against human prion proteins in prp0/0 mice. *Molecular Medicine*, **2**, 725-734.

Krausslich, H.G. (1991) Human immunodeficiency virus proteinase dimer as component of the viral polyprotein prevents particle assembly and viral infectivity. *Proc. Natl Acad. Sci. U S A*, **88**, 3213-3217.

Kretzschmar, H.A. (1993) Human prion diseases (spongiform encephalopathies). *Arch. Virol. Suppl.*, **7**, 261-293.

Kuwahara, C., Takeuchi, A.M., Nishimura, T., Haraguchi, K., Kubosaki, A., Matsumoto, Y., Saeki, K., Matsumoto, Y., Yokoyama, T., Itohara, S. and Onodera, T. (1999) Prions prevent neuronal cell-line death. *Nature*, **400**, 225-226.

Laing, J.G., Robertson, M.W., Gritzmacher, C.A., Wang, J.L. and Liu, F.T. (1989) Biochemical and immunological comparisons of carbohydrate-binding protein 35 and an IgE-binding protein. *J. Biol. Chem.*, **264**, 1097-1010.

Landowski, T.H., Dratz, E.A. and Starkey, J.R. (1995) Studies of the structure of the metastasis-associated 67 kDa laminin binding protein: Fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein. *Biochemistry*, **34**, 11276-11287.

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

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

Lee, K.S., Magalhaes, A.C., Zanata, S.M., Brentani, R.R., Martins, V.R. and Prado, M.A. (2001) Internalization of mammalian fluorescent cellular prion protein and N-terminal deletion mutants in living cells. *Journal of neurochemistry*, **79**, 79-87.

Lesot, H., Kühl, U. and von der Mark, K. (1983) Isolation of a laminin binding protein from muscle cell membranes. *EMBO J.*, **2**, 861-865.

Leucht, C., Simoneau, S., Rieger, R., Rey, C., Lasmézas, C.I. and Weiss, S. (2003) 37-kDa LRP antisense RNA, small interfering RNAs and the anti-LRP/LR specific antibody W3 repress PrPres accumulation in Scrapie infected neuronal cells. *EMBO Reports*, **4**, 290–295.

Leucht, C. and Weiss, S. (2002) In BSE-Wahnsinn und Wirklichkeit (Ed. Brem, M. & Müller, M.) Vol 87, 39-54. Nova Acta Leopoldina, Halle.

Li, A., Sakaguchi, S., Shigematsu, K., Atarashi, R., Roy, B.C., Nakaoke, R., Arima, K., Okimura, N., Kopacek, J. and Katamine, S. (2000) Physiological expression of the gene for PrP-like protein, PrPLP/Dpl, by brain endothelial cells and its ectopic expression in neurons of PrP-deficient mice ataxic due to Purkinje cell degeneration. *Am J. Pathol.*, **157**, 1447-1452.

Liljestrom, P. and Garoff, H. (1991a) Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.*, **65**, 147-154.

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

Liu, H., Farr, J.S., Ulyanov, N.B., Llinas, M., Marqusee, S., Groth, D., Cohen, F.E., Prusiner, S.B. and James, T.L. (1999) Solution structure of Syrian hamster prion protein rPrP(90-231). *Biochemistry*, **38**, 5362-5377.

Lledo, P.M., Tremblay, P., DeArmond, S.J., Prusiner, S.B. and Nicoll, R.A. (1996) Mice deficient for prion protein exhibit normal neuronal excitability and synaptic transmission in the hippocampus. *Proc. Natl. Acad. Sci. USA*, **93**, 2403-2407.

Lopez Garcia, F., Zahn, R., Riek, R. and Wuthrich, K. (2000) NMR structure of the bovine prion protein. *Proc. Natl Acad. Sci. U S A*, **97**, 8334-8399.

Lopez-Ribot, J.L., Casanova, M., Monteagudo, C., Sepulveda, P. and Martinez, J.P. (1994) Evidence for the presence of a high-affinity laminin receptor-like molecule on the surface of Candida albicans yeast cells. *Infect. Immun.*, **62**, 742-746.

Lorains, J.W., Henry, C., Agbamu, D.A., Rossi, M., Bishop, M., Will, R.G. and Ironside, J.W. (2001) Variant Creutzfeldt-Jakob disease in an elderly patient. *Lancet*.

Lu, B.Y. and Chang, J.Y. (2001) Isolation of isoforms of mouse prion protein with PrP(SC)-like structural properties. *Biochemistry*, **40**, 13390-13396.

Ludwig, G.V., Kondig, J.P. and Smith, J.F. (1996) A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J. Virol.*, **70**, 5592-5599.

Lugaresi, E., Medori, R., Montagna, P., Baruzzi, A., Cortelli, P., Lugaresi, A., Tinuper, P., Zucconi, M. and Gambetti, P. (1986) Fatal familial insomnia and dysautonomia with selective degeneration of thalamic nuclei. *N. Engl. J. Med.*, **315**, 997-1003.

Ma, J. and Lindquist, S. (2002) Conversion of PrP to a Self-Perpetuating PrPSc-like Conformation in the Cytosol. *Science*, **298**,1785-1788

Magnifico, A., Tagliabue, E., Buto, S., Ardini, E., Castronovo, V., Colnaghi, M.I. and Menard, S. (1996) Peptide G, containing the binding site of the 67-kDa laminin receptor, increases and stabilizes laminin binding to cancer cells. *J. Biol. Chem.*, **271**, 31179-31184.

Malinoff, H.L. and Wicha, M.S. (1983) Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. *J. Cell. Biol.*, **96**, 1475-1479.

Mallucci, G.R., Ratte, S., Asante, E.A., Linehan, J., Gowland, I., Jefferys, J.G.R. and Collinge, J. (2002) Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.*, **21**, 202-210

Manetto, V., Medori, R., Cortelli, P., Montagna, P., Tinuper, P., Baruzzi, A., Rancurel, G., Hauw, J., Vanderhaeghen, J., Mailleux, P. and al., e. (1992) Fatal familial insomnia: clinical and pathologic study of five new cases. *Neurology*, **42**, 312-319.

Mange, A., Nishida, N., Milhavet, O., McMahon, H.E.M., Casanova, D. and Lehmann, S. (2000) Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. *J. Virol.,* **74**, 3135-3140

Manson, J.C., Clarke, A.R., Hooper, M.L., Aitchison, L., McConnell, I. and Hope, J. (1994) 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Mol. Neurobiol.*, **8**, 121-127.

Martins, V.R. (1999) A receptor for infectious and cellular prion protein. *Braz. J. Med. Biol. Res.*, **32**, 853-859.

Martins, V.R., Graner, E., Garcia, A.J., de, S.S., Mercadante, A.F., Veiga, S.S., Zanata, S.M., Neto, V.M. and Brentani, R.R. (1997) Complementary hydropathy identifies a cellular prion protein receptor. *Nat. Med.*, **3**, 1376-1382.

Maxfield, F.R. and Mayor, S. (1997) Cell surface dynamics of GPI-anchored proteins. *Adv. Exp. Med. Biol.*, **419**, 355-364.

McKinley, M.P., Meyer, R.K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A. and Prusiner, S.B. (1991) Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis. *J. Virol.*, **65**, 1340-1351.

Mecham, R.P. (1991) Receptors for laminin on mammalian cells. *FASEB J.*, **5**, 2538-2546.

Medori, R., Montagna, P., Tritschler, H., LeBlanc, A., Cortelli, P., Tinuper, P., Lugaresi, E. and Gambetti, P. (1992) Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. *Neurology*, **42**, 669-670.

Melnick, M.B., Noll, E. and Perrimon, N. (1993) The Drosophila stubarista phenotype is associated with a dosage effect of the putative ribosome-associated protein D-p40 on spineless. *Genetics*, **135**, 553-564.

Menard, S., Castronovo, V., Tagliabue, E. and Sobel, M.E. (1997) New insights into the metastasis-associated 67 kD laminin receptor. *Journal of Cellular Biochemistry.*

Meyer, R.K., Lustig, A., Oesch, B., Fatzer, R., Zurbriggen, A. and Vandevelde, M. (2000) A Monomer-Dimer Equilibrium of a cellular prion protein (PrPc) not observed with recobinant PrP. *J. Biol. Chem.*, **275**, 38081-38087.

Meyer, R.K., McKinley, M.P., Bowman, K.A., Braunfeld, M.B., Barry, R.A. and Prusiner, S.B. (1986) Separation and properties of cellular and scrapie prion proteins. *Proc. Natl. Acad. Sci. USA*, **83**, 2310-2314.

Moore, R.C., Lee, I.Y., Silverman, G.L., Harrison, P.M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S.H., Chishti, M.A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A.F.A., Katamine, S., Carlson, G.A., Cohen, F.E., Prusiner, S.B., Melton, D.W., Tremblay, P., Hood, L.E. and Westaway, D. (1999) Ataxia in Prion Protein (PrP)-deficient Mice is Associated with Upregulation of the Novel PrP-like Protein Doppel. *J. Mol. Biol.*, **292**, 797-817.

Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J.M. and Kellermann, O. (2000) Signal transduction through prion protein. *Science*, **289**, 1925-1928.

Murtomaki, S., Trenkner, E., Wright, J.M., Saksela, O. and Liesi, P. (1995) Increased proteolytic activity of the granule neurons may contribute to neuronal death in the weaver mouse cerebellum. *Dev. Biol.*, **168**, 635-648.

Nunziante, M., Gilch, S. and Schätzl, H.M. (2002) Essential role of the prion protein N-terminus in subcellular trafficking and half-life of PrPc. *J. Biol. Chem.*, **278**, 3726-3734

Ochieng, J., Platt, D., Tait, L., Hogan, V., Raz, T., Carmi, P. and Raz, A. (1993) Structure-function relationship of a recombinant human galactoside-binding protein. *Biochemistry*, **32**, 4455-4460.

Oesch, B., Westaway, D. and Prusiner, S.B. (1991) Prion protein genes: evolutionary and functional aspects. In Chesebro, B.W. (ed.), *Current topics in microbiology and immunology. Transmissible spongiform encephalopathies: scrapie, BSE and related disorders.* Springer-Verlag, Berlin, pp. 109-124.

Oesch, B., Westaway, D., Waelchli, M., McKinley, M.P., Kent, S.B., Aebersold, R., Barry, R.A., Tempst, P., Teplow, D.B., Hood, L.E., Prusiner, S.B. and Weissmann, C. (1985) A cellular gene encodes scrapie PrP 27-30 protein. *Cell*, **40**, 735-746.

Ouzonis, C., Kyrpides, N. and Sander, C. (1995) Novel protein families in archaean genomes. *Nucleic Acids Res*, **23**, 565-570.

Owen, F., Poulter, M., Collinge, J. and Crow, T.J. (1990) Codon 129 changes in the prion protein gene in Caucasians. *Am J Hum Genet*, **46**, 1215-1216.

Palmer, M.S., Dryden, A.J., Hughes, J.T. and Collinge, J. (1991) Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. *Nature*, **352**, 340-342.

Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E. and Prusiner, S.B. (1993) Conversion of a-helices into

ß-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. USA*, **90**, 10962-10966.

Panaretou, B. and Piper, P. (1996) Isolation of yeast plasma membranes. In Evans, I.H. (ed.), *Methods in Molecular Biology; Yeast Protocols*. Humana Press, Totowa, Vol. 53, pp. 117-121.

Pauly, P.C. and Harris, D.A. (1998) Copper stimulates endocytosis of the prion protein. *Journal of biological chemistry*, **273**, 33107-33110.

Peretz, D., Williamson, R.A., Kaneko, K., Vergara, J., Leclerc, E., Schmitt Ulms, G., Mehlhorn, I.R., Legname, G., Wormald, M.R., Rudd, P.M., Dwek, R.A., Burton, D.R. and Prusiner, S.B. (2001) Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. *Nature*, **412**, 739-743.

Philips, N.B., J; Ferguson-Smith, M. (2000) BSE inquiry report. Stationary Office, London, Vol. 2: Science

Pley, U. and Parham, P. (1993) Clathrin: its role in receptor-mediated vesicular transport and specialized functions in neurons. *Crit. Rev. Biochem. Mol. Biol.*, **28**, 431-464.

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

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

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

Prusiner, S.B. (1991) Molecular biology of prion diseases. *Science*, **252**, 1515-1522.

Prusiner, S.B. (1993) Genetic and infectious prion diseases. *Arch. Neurol.*, **50**, 1129-1153.

Prusiner, S.B. (1998) Prions. *Proc. Natl Acad. Sci. USA*, **95**, 13363-13383.

Prusiner, S.B., Bolton, D.C., Groth, D.F., Bowman, K.A., Cochran, S.P. and McKinley, M.P. (1982) Further purification and characterization of scrapie prions. *Biochemistry*, **21**, 6942-6950.

Prusiner, S.B., Groth, D.F., Bolton, D.C., Kent, S.B. and Hood, L.E. (1984) Purification and structural studies of a major scrapie prion protein. *Cell*, **38**, 127-134.

Prusiner, S.B., McKinley, M.P., Groth, D.F., Bowman, K.A., Mack, N.I., Cochran, S.P. and Masiarz, F.R. (1981) Scrapie agent contains a hydrophobic protein. *Proc. Natl. Acad. Sci. USA*, **78**, 6675-6679.

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

Rao, C.N., Castronovo, V., Schmitt, M.C., Wewer, U.M., Claysmith, A.P., Liotta, L.A. and Sobel, M.E. (1989) Evidence for a precursor of the high-affinity metastasis-associated murine laminin receptor. *Biochemistry*, **28**, 7476-7486.

Rao, N.C., Barsky, S.H., Terranova, V.P. and Liotta, L.A. (1983) Isolation of a tumor cell laminin receptor. *Biochem. Biophys. Res. Commun.*, **111**, 804-808.

Raymond, G.J., Hope, J., Kocisko, D.A., Priola, S.A., Raymond, L.D., Bossers, A., Ironside, J., Will, R.G., Chen, S.G., Petersen, R.B., Gambetti, P., Rubenstein, R., Smits, M.A., Lansbury, P.J. and Caughey, B. (1997) Molecular assessment of the potential transmissibilities of BSE and scrapie to humans [see comments]. *Nature*, **388**, 285-288.

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

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

Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R. and Wuthrich, K. (1996) NMR structure of the mouse prion protein domain PrP(121-231). *Nature*, **382**, 180-182.

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

Rogers, M., Serban, D., Gyuris, T., Scott, M., Torchia, T. and Prusiner, S.B. (1991) Epitope mapping of the Syrian hamster prion protein utilizing chimeric and mutant genes in a vaccinia virus expression system. *J. Immunol.*, **147**, 3568-3574.

Romanos, M. (1995) Advances in the uses of *Pichia pastoris* for high-level gene expression. *Curr. Opin. Biotech.*, **6**, 527-533.

Rosenthal, E.T. and Wordeman, L. (1995) A protein similar to the 67 kDa laminin binding protein and p40 is probably a component of the translational machinery in Urechis caupo oocytes and embryos. *J. Cell. Sci.*, **108**, 245-256.

Rossi, D., Cozzio, A., Flechsig, E., Klein, M.A., Rulicke, T., Aguzzi, A. and Weissmann, C. (2001) Onset of ataxia and Purkinje cell loss in PrP null mice inversely correlated with Dpl level in brain. *EMBO J.*, **20**, 694-702.

Rudd, P.M., Endo, T., Colominas, C., Groth, D., Wheeler, S.F., Harvey, D.J., Wormald, M.R., Serban, H., Prusiner, S.B., Kobata, A. and Dwek, R.A. (1999) Glycosylation differences between the normal and pathogenic prion protein isoforms. *Proc. Natl. Acad. Sci. USA*, **96**, 13044-13049.

Safar, J., Roller, P.P., Gajdusek, D.C. and Gibbs, C.J.J. (1993) Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. *J. Biol. Chem.*, **268**, 20276-20284.

Sakaguchi, S., Katamine, S., Nishida, N., Moriuchi, R., Shigematsu, K., Sugimoto, T., Nakatani, A., Kataoka, Y., Houtani, T., Shirabe, S., Okada, H., Hasegawa, S., Miyamoto, T. and Noda, T. (1996) Loss of cerebellar Purkinje cells in aged mice homozygous for a disrupted PrP gene. *Nature*, **380**, 528-531.

Salas, P.J., Ponce, M.I., Brignoni, M. and Rodriguez, M.L. (1992) Attachment of Madin-Darby canine kidney cells to extracellular matrix: role of a laminin binding protein related to the 37/67 kDa laminin receptor in the development of plasma membrane polarization. *Biol. Cell.*, **75**, 197-210.

Sato, M., Kinoshita, K., Kaneda, Y., Saeki, Y., Iwamatsu, A. and Tanaka, K. (1996) Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). *Biochem. Biophys. Res. Commun.*, **229**, 896-901.

Sato, M., Saeki, Y., Tanaka, K. and Kaneda, Y. (1999) Ribosome-associated protein LBP/p40 binds to S21 protein of 40S ribosome: analysis using a yeast two-hybrid system. *Biochem. Biophys. Res. Commun.*, **256**, 385-390.

Schätzl, H.M., Da-Costa, M., Taylor, L., Cohen, F.E. and Prusiner, S.B. (1995) Prion protein gene variation among primates. *J. Mol. Biol.*, **245**, 362-374.

Schmid, S.L. (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.*, **66**, 511-548.

Schreuder, B.E. (1994) Animal spongiform encephalopathies--an update. Part 1. Scrapie and lesser known animal spongiform encephalopathies. *Vet. Q.*, **16**, 174-181.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S.J., Westaway, D. and Prusiner, S.B. (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell*.

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

Shaked, G.M., Fridlander, G., Meiner, Z., Taraboulos, A. and Gabizon, R. (1999) Protease-resistant and detergent-insoluble prion protein is not necessarily associated with prion infectivity. *J. Biol. Chem.*, **274**, 17981-17986.

Shmakov, A.N., Bode, J., Kilshaw, P.J. and Ghosh, S. (2000) Diverse patterns of expression of the 67-kD laminin receptor in human small intestinal mucosa: Potential binding sites for prion proteins? *J. Pathol.*, **191**, 318-322.

Shmerling, D., Hegyi, I., Fischer, M., Blaettler, T., Brandner, S., Goetz, J., Ruelicke, T., Flechsig, E., Cozzio, A., von Mering, C., Hangartner, C., Aguzzi, A. and Weissmann, C. (1998) Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell*, **93**, 203-214.

Shyng, S.L., Heuser, J.E. and Harris, D.A. (1994) A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. *J. Cell. Biol.*, **125**, 1239-1250.

Shyng, S.L., Lehmann, S., Moulder, K.L. and Harris, D.A. (1995a) Sulfated glycans stimulate endocytosis of the cellular isoform of the prion protein, PrP^{C}, in cultured cells. *J. Biol. Chem.*, **270**, 30221-30229.

Shyng, S.L., Moulder, K.L., Lesko, A. and Harris, D.A. (1995b) The N-terminal domain of a glycolipid -anchored prion protein is essential for its endocytosis via clathrin-coated pits. *J. Biol. Chem.*, **270**, 14793-14800.

Silverman, G.L., Qin, K., Moore, R.C., Yang, Y., Mastrangelo, P., Tremblay, P., Prusiner, S.B., Cohen, F.E. and Westaway, D. (2000) Doppel is an N-glycosylated, glycosylphosphatidylinositol-anchored protein. Expression in testis and ectopic production in the brains of Prnp(0/0) mice predisposed to Purkinje cell loss. *J. Biol. Chem.*, **275**, 26834-26841.

Simoneau, S., Haïk, S., Leucht, C., Dormont, D., Deslys, J.-P., Weiss, S. and Lasmézas, C.I. (2003) Different Isoforms of the Non-Integrin Laminin Receptor are Present in Mouse Brain and Bind PrP. *Biol. Chem.*, **384**, 243–246

Sohn, H.J., Kim, J.H., Choi, K.S., Nah, J.J., Joo, Y.S., Jean, Y.H., Ahn, S.W., Kim, O.K., Kim, D.Y. and Balachandran, A. (2002) A case of chronic wasting disease in an elk imported to Korea from Canada. *J Vet Med Sci*, **64**, 855-858.

Sondermann, H., Becker, T., Mayhew, M., Wieland, F. and Hartl, F.U. (2000) Characterization of a receptor for heat shock protein 70 on macrophages and monocytes. *Biol. Chem.*, **381**, 1165-1174.

Stahl, N., Baldwin, M.A., Teplow, D.B., Hood, L., Gibson, B.W., Burlingame, A.L. and Prusiner, S.B. (1993) Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry*, **32**, 1991-2002.

Stahl, N., Borchelt, D.R., Hsiao, K. and Prusiner, S.B. (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell*, **51**, 229-240.

Stephenson, D.A., Chiotti, K., Ebeling, C., Groth, D., DeArmond, S.J., Prusiner, S.B. and Carlson, G.A. (2000) Quantitative trait loci affecting prion incubation time in mice. *Genomics*, **69**, 47-53.

Stern, K. (1939) Severe dementia associated with bilateral symmetrical degeneration of the thalamus. *Brain*, **62**, 157-171.

Taraboulos, A., Rogers, M., Borchelt, D.R., McKinley, M.P., Scott, M., Serban, D. and Prusiner, S.B. (1990) Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proc. Natl. Acad. Sci. USA*, **87**, 8262-8266.

Taratuto, A., Piccardo, P., Reich, E., Chen, S., Sevlever, G., Schultz, M., Luzzi, A., Rugiero, M., Abecasis, G., Endelman, M., Garcia, A., Capellari, S., Xie, Z., Lugaresi, E., Gambetti, P., Dlouhy, S. and Ghetti, B. (2002) Insomnia associated with thalamic involvement in E200K Creutzfeldt-Jakob disease. *Neurology,* **58**, 362-367.

Telling, G.C., Scott, M., Mastrianni, J., Gabizon, R., Torchia, M., Cohen, F.E., DeArmond, S.J. and Prusiner, S.B. (1995) Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell*, **83**, 79-90.

Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M., Rülicke, T., Moser, M., Oesch, B., McBride, P. and Manson, J.C. (1996) Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature*, **380**, 639-642.

Tremblay, P., Meiner, Z., Galou, M., Heinrich, C., Petromilli, C., Lisse, T., Cayetano, J., Torchia, M., Mobley, W., Bujard, H., Dearmond, S.J. and Prusiner, S.B. (1998) Doxycycline control of prion protein transgene expression modulates priori disease in mice. *Proceedings of the National Academy of Sciences of the United States of America*.

Tubulekas, I., Berglund, P., Fleeton, M. and Liljestrom, P. (1997) Alphavirus expression vectors and their use as recombinant vaccines: a minireview. *Gene*, **190**, 191-195.

Turk, E., Teplow, D.B., Hood, L.E. and Prusiner, S.B. (1988) Purification and properties of the cellular and scrapie hamster prion proteins. *Eur. J. Biochem.*, **176**, 21-30.

van Duijn, C.M., Delasnerie-Laupretre, N., Masullo, C., Zerr, I., de Silva, R., Wientjens, D.P., Brandel, J.P., Weber, T., Bonavita, V., Zeidler, M., Alperovitch, A., Poser, S., Granieri, E., Hofman, A. and Will, R.G. (1998) Case-control study of risk factors of Creutzfeldt-Jakob disease in Europe during 1993-95. European Union (EU) Collaborative Study Group of Creutzfeldt-Jakob disease (CJD). *Lancet*, **351**, 1081-1085.

Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S.J., Smart, E.J., Anderson, R.G., Taraboulos, A. and Prusiner, S.B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl Acad. Sci. USA*, **93**, 14945-14949.

Vleurick, L., Kuhn, E.R., Decuypere, E. and Van, V.P. (1999) Isolation of plasma membranes and Golgi apparatus from a single chicken liver homogenate. *J. Cell. Biochem.*, **72**, 349-355.

Volkel, D., Blankenfeldt, W. and Schomburg, D. (1998) Large-scale production, purification and refolding of the full-length cellular prion protein from Syrian golden hamster in Escherichia coli using the glutathione S-transferase fusion system. *Eur J Biochem*, **251**, 462-471.

Wang, K.S., Kuhn, R.J., Strauss, E.G., Ou, S. and Strauss, J.H. (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J. Virol.*, **66**, 4992-5001.

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

Warwicker, J. (1997) Species Barriers in a Model for specific prion protein dimerization. *Biochem. Biophys. Res. Commun.*, **232**, 508-512.

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

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

Weiss, S., Rieger, R., Edenhofer, F., Fisch, E. and Winnacker, E.-L. (1996) Recombinant prion protein rPrP27-30 from Syrian Golden Hamster reveals proteinase K sensitivity. *Biochem. Biophys. Res. Commun.*, **219**, 173-179.

Weissmann, C. (1996) PrP effects clarified. *Curr. Biol.*, **6**, 1359.

Weissmann, C. (1999) Molecular genetics of transmissible spongiform encephalopathies. *J. Biol. Chem.*, **274**, 3-6.

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

Wells, G.A., Scott, A.C., T, J.C., F, G.R., D, H.R., M, J., M, D. and R, B. (1987) A novel progressive spongiform encephalopathy in cattle. *Vet Rec*, **121**, 419-420.

Wewer, U.M., Liotta, L., Jaye, M., Ricca, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudry, M. and Sobel, M.E. (1986) Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. *Proc. Natl. Acad. Sci. USA*, **83**, 7137-7141.

Wewer, U.M., Taraboletti, G., Sobel, M.E., Albrechtsen, R. and Liotta, L.A. (1987) Role of laminin receptor in tumor cell migration. *Cancer Research*.

Wilesmith, J.W., Ryan, J.B. and Atkinson, M.J. (1991) Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec*, **128**, 199-203.

Will, R.G. (2002) Variant Creutzfeldt-Jakod disease. *Acta Neurobiol. Exp*., **62**, 167-173

Will, R.G., Cousens, S.N., Farrington, C.P., Smith, P.G., Knight, R.S. and Ironside, J.W. (1999) Deaths from variant Creutzfeldt-Jakob disease. *Lancet*, **353**, 979.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A. and Smith, P.G. (1996) A new variant of Creutzfeldt-Jakob disease in the UK [see comments]. *Lancet*, **347**, 921-925.

Williams, E.S. and Young, S. (1980)

Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wildl Dis*, **16**, 89-98.

Wopfner, F., Weidenhofer, G., R, S., von Brunn, A., Gilch, S., Schwarz, T.F., Werner, T. and Schatzl, H.M. (1999) Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J. Mol. Biol.*, **289**, 1163-1178.

Yang, R.Y., Hsu, D.K. and Liu, F.T. (1996) Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc. Natl Acad. Sci. USA*, **93**, 6737-6742.

Yow, H., Wong, J.M., Chen, H.S., Lee, C., Steele, G.D.J. and Chen, L.B. (1988) Increased mRNA expression of a laminin-binding protein in human colon carcinoma: complete sequence of a full-length cDNA encoding the protein. *Proc. Natl. Acad. Sci. USA*, **85**, 6394-6398.

Zahn, R., Liu, A., Luhrs, T., Riek, R., von Schroetter, C., Lopez Garcia, F., Billeter, M., Calzolai, L., Wider, G. and Wuthrich, K. (2000) NMR solution structure of the human prion protein. *Proc. Natl Acad. Sci. USA*, **97**, 145-150.

Zanata, S.M., Lopes, M.H., Mercadante, A.F., Hajj, G.N.M., Chiarini, L.B., Nomizo, R., Freitas, A.R.O., Cabral, A.L.B., Lee, K.S., Juliano, M.A., De Oliveira, E., Jachieri, S.G., Burlingame, A., Huang, L., Linden, R., Brentani, R.R. and Martins, V.R. (2002) Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J.*, **21**, 3307-3316

Zigas, V. and Gajdusek, D.C. (1957) Kuru: Clinical Study of a New Syndrome Resembling Paralysis Agitans in Natives of the Eastern Highlands of Australian New Guinea. *Medical Journal of Australia*, **II**, 745-754.

Abbreviations

aa	amino acid
Ab	antibody
AD	Alzheimer disease
AmB	amphotericin B
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 albumin
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
DWS	aspartic acid-tryptophan-serine
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
FITC	fluorescein isothiocyanate
FFI	fatal familial insomnia
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	kilobases
kDa	kiloDalton
LR	laminin receptor
LRP	laminin receptor precursor
LRS	lymphoreticular system
mAb	monoclonal Ab
min	minutes
moPrP	mouse PrP
mRNA	messenger RNA
M	molar
MW	molecular weight
NMR	nuclear magnetic resonance
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	rounds per minute

SDS	sodium dodecyl sulfate
SFV	Semliki Forest virus
siRNA	short interfering RNA
SP54	pentosan polysulfate
TEMED	N,N,N',N'-tetramethylethylendiamine
TGN	trans-golgi-network
TMAO	trimethylamine-*N*-oxide
TMD	transmembrane domain
TSE	transmissible spongiforme encephalopathy
UK	United Kingdom
vCJD	new variant CJD
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Curriculum vitae

Christoph Leucht
Geltingerstr. 10
81379 München
Telefon: 089/44770463
E-Mail: leucht@topforscher.de

Persönliche Daten

Geburtsdatum	16.01.1973
Geburtsort	Frankfurt/Main
Familienstand	ledig
Nationalität	deutsch

Schulische Laufbahn

Grundschule in Frankfurt/Main
Gymnasium (Liebigschule) in Frankfurt/Main
1992 Erlangung der allgemeinen Hochschulreife

Wehrdienst

Ausbildung zum Sanitätssoldat in Rennerod. Dienst im Sänitätsbereich der Steubenkaserne in Gießen.

Studium und Diplom

Studiengang der Biologie an der Johann-Wolfgang-Goethe Universität in Frankfurt/Main

ab 1995 Vordiplom im Studiengang Biologie
Hauptstudium der Biochemie an der Johann-

Wolfgang-Goethe Universität in Frankfurt/Main

Diplomprüfungen im Studiengang der Biochemie:

Biochemie

Biophysikalische Chemie

Pharmakologie

März-Oktober 1998

Diplomarbeit am Max-Planck-Institut für Biophysik in der Abteilung für Molekulare Membranbiologie unter Leitung von Prof.Dr.Hartmut Michel:

Studien zur Expression von Antikörper-F_v-Fragmenten in der Hefe *Pichia pastoris*

Promotion

Seit 1999 Promotion am Institut für Biochemie/Genzentrum der LMU-München in der Arbeitsgruppe für Prionenforschung unter Leitung von Priv.-Doz. Dr. Stefan Weiß.

Thema der Promotion:

The role of the 37kDa/67kDa laminin receptor in the cellular metabolism of the prion protein