Insights into the physiological function of cellular prion protein

Abstract

Prions have been extensively studied since they represent a new class of infectious agents in which a protein, PrPsc (prion scrapie), appears to be the sole component of the infectious particle. They are responsible for transmissible spongiform encephalopathies, which affect both humans and animals. The mechanism of disease propagation is well understood and involves the interaction of PrPsc with its cellular isoform (PrPc) and subsequently abnormal structural conversion of the latter. PrPc is a glycoprotein anchored on the cell surface by a glycosylphosphatidylinositol moiety and expressed in most cell types but mainly in neurons. Prion diseases have been associated with the accumulation of the abnormally folded protein and its neurotoxic effects; however, it is not known if PrPc loss of function is an important component. New efforts are addressing this question and trying to characterize the physiological function of PrPc. At least four different mouse strains in which the PrP gene was ablated were generated and the results regarding their phenotype are controversial. Localization of PrPc on the cell membrane makes it a potential candidate for a ligand uptake, cell adhesion and recognition molecule or a membrane signaling molecule. Recent data have shown a potential role for PrPc in the metabolism of copper and moreover that this metal stimulates PrPc endocytosis. Our group has recently demonstrated that PrPc is a high affinity laminin ligand and that this interaction mediates neuronal cell adhesion and neurite extension and maintenance. Moreover, PrPc-caveolin-1 dependent coupling seems to trigger the tyrosine kinase Fyn activation. These data provide the first evidence for PrPc involvement in signal transduction.

Introduction

The cellular prion protein was first identified in experiments conducted in an attempt to find the exogenous nucleic acid component of the infectious agent responsible for neurodegenerative diseases called transmissible spongiform encephalopathies (TSE). This agent was partially purified from the brain of affected animals. An insoluble protein of 33-35 kDa designated PrPsc (prion scrapie) which generates a 27-30-kDa form after protease treatment was identified as the major component of the agent. Amino acid
sequencing of the amino-terminal region from the purified protein allowed the synthesis of an isocoding mixture of nucleotides that was subsequently used to identify prion cDNA clones. The amino-terminal peptide was also used to produce polyclonal antibodies. These reagents allowed the identification of a chromosomai gene and a host cellular protein, PrPc, expressed in a variety of neuronal and nonneuronal tissues independently of the infection by scrapie or any other TSE agent (1).

The participation of PrPc is absolutely necessary for infection since animals in which the PrP gene has been ablated are totally resistant to the infection (2). PrPc and PrPsc have the same amino acid composition, although the α-helix content of PrPc is about 40%, with less than 10% β-sheets conformation. In contrast, PrPsc shows about 50% of its structure as a β-sheet. Therefore, it was proposed that gain of infectivity is a consequence of conformational modification of PrPc by PrPsc (1).

PrPc protein is highly conserved among species; the similarity is about 85 to 97% among mammals (3) and the comparison between primates and humans showed amino acid identity ranging from 92.9 to 99.6% (4). PrPc has also been described in chickens (3) and turtles (5). The entire open reading frame of all known PrP genes is located in a single exon which codes for a protein of approximately 250 amino acids (3). A signal sequence of 22 amino acids is present at the amino-terminal (6) and a 23-amino acid signal sequence encoding for attachment to a glycosylphosphatidylinositol (GPI) anchors at the carboxy-terminal sequence (7).

The cellular prion protein has some characteristics that make it a very interesting molecule and its conservation among species strongly suggests its relevance in physiological processes. Moreover, the role of PrPc in TSE is viewed as gain of function due to accumulation of a new PrPc isoform, PrPsc. However, it is still possible that prion diseases could be mediated, at least in part, by loss of function (8,9).

During the last few years several research groups have been working with different models aiming to understand the physiological function of PrPc. Since this protein is a cell surface molecule its role could be related to ligand uptake, cell adhesion and recognition or cell signaling (10). Herein we intend to present an overview of the possible functions of cellular prion protein.

**Generation of PrP-deleted animals**

The generation of animals in which the gene that codes for a protein of interest is deleted, is a very interesting approach to study the unknown function of this gene in cellular or animal physiology.

Charles Weismann’s group generated the first PrPc gene (Prnp)-deleted mouse (PrPc−/− Zrch) in 1992 by homologous recombination replacing the Prnp open reading frame with the neomycin phosphotransferase gene under control of the herpes simplex virus thymidine kinase promoter (11). They performed learning, immunologic and anatomical tests in order to determine if PrPc is essential in some of these processes. The structure of PrPc−/− mouse brain was normal and no detectable effect on the level of lymphocyte surface MHC class I and II antigen markers was observed. Behavior analysis with these mice included three tests: swimming navigation to find a submerged platform, Y-maze discrimination and two-way avoidance with shock sensitivity. Normal and PrPc−/− mice showed a poor but overall significant learning performance, which is typical for many mouse strains, but no significant differences between them were observed. These results were somehow disappointing since due to the high conservation of PrP among species and its brain expression, the research community was expecting important physiological deficiencies in those animals. However, at least two possibilities
can be proposed: the resulting defect is so subtle that a selective disadvantage may emerge only after many generations, or the protein function is redundant (11).

One year later, the same group observed that these mice devoid of PrP are completely protected against scrapie disease, at least up to 13 months after inoculation. Moreover, even heterozygous Prnp$^{+/}$ mice are partially protected, inasmuch as 9 of 10 scrapie-inoculated animals showed signs of scrapie only 253-322 days after inoculation but are still alive after 322 days, while all Prnp$^{+/}$ controls died within about 180 days (2). These observations definitely prove that the development of scrapie symptoms and pathology is strictly dependent on the presence of PrPc and also that the expression level of PrPc is inversely related to incubation time and disease progression.

A second Prnp$^{-/-}$ line was created (Npu) two years later by a different targeting strategy but again it was impossible to obtain information about the normal function of PrPc since the line showed normal development (12).

Later, the application of specific behavioral tests characterized some abnormalities imputable to the lack of PrPc in Zrch animals (the first generated PrPc$^{-/-}$ mice). Tobler and co-workers (13) described an alteration in circadian rhythms which correlated the circadian regulation function of PrPc with fatal familial insomnia (a hereditary disease associated with specific mutations of the Prnp gene). A weakened GABA-A receptor-mediated fast inhibition and impaired long-term potentiation (14) were also described and could be involved in the epileptic activity seen in Creutzfeldt-Jakob disease. Prusiner’s group (15) did not reproduce these results; however, we observed that these Prnp$^{-/-}$ mice have an increased sensitivity to seizures in four different epileptogenical models (16).

In an attempt to analyze different behavioral tasks of mice devoid of PrPc our group reported an increased locomotor activity in Prnp$^{-/-}$ Zrch mice, but normal inhibitory avoidance learning and anxiety (17).

In 1996 Sakaguchi and co-workers (18) developed a third line of mice homozygous for a disrupted Prnp gene (Ngsk) and observed that these animals grew normally after birth, but at about 70 weeks of age all began to show progressive symptoms of ataxia. The brain of mice with neurological symptoms presented considerable atrophy of the cerebellum due to an extensive loss of Purkinje cells.

One year later another Prnp$^{-/-}$ mouse line was generated (Rcm0) and also developed a late onset fatal ataxia (19). Thus, it was suggested that PrPc has a role in the long-term survival of Purkinje neurons. However, it was puzzling why Ngsk and Rcm0 mice developed a fatal ataxia while two other lines of Prnp$^{-/-}$ mice did not exhibit any extensive CNS dysfunction.

In order to determine the role of PrPc in this phenotype, Moore’s group (19) analyzed PrPc-related genes. Since most of these related genes are localized in clusters they sequenced large cosmid clones containing the Prnp gene and found a novel PrP-like gene named Doppel (German for double and also meaning downstream prion-like protein). The coding region for Doppel (Prnd) is located 16 kb downstream from the Prnp gene and two major transcripts of 1.7 and 2.7 kb as well as an unusual chimeric transcript, generated by intergenic splicing with Prnp, are produced. Interestingly, the chimeric transcript is up-regulated in the Ngsk and Rcm0 strains of PrPc$^{-/-}$ mice that developed ataxia but not in Zrch or Npu strains with a normal phenotype. The authors suggest that Doppel overexpression may provoke neurodegeneration (19).

The construction of a mouse with a prion transgene rescues the ataxia and Purkinje cell degeneration phenotype in Ngsk Prnp$^{-/-}$ mice (20), suggesting that PrPc and Doppel proteins might compete for a common re-
ceptor protein (19). A few years ago our group described a receptor for prion protein (21) and we speculated that PrPc binding to this receptor should participate in PrPc internalization and signaling. The evaluation of PrPc. Doppel, a common ligand and their interaction with other proteins might allow the characterization of PrPc physiological function. This subject will be discussed later in this article.

**PrPc as a copper uptake protein**

There is increasing evidence supporting a functional role for PrPc in copper metabolism. Several studies have indicated that PrPc can bind copper. First, it was possible to isolate PrPc from hamster brain on a copper affinity column (22). Moreover, purified recombinant PrPc, as well as synthetic PrPc-derived peptides, bind copper ions with micromolar affinity through four histidine-containing peptide repeats in the amino-terminal half of the protein (23-25). The repeated region contains five or six octapeptide tandem motifs of the general form P(H/G)GGGWGQ and is highly conserved among mammalian PrPc, while chicken PrPc has a similar region of eight hexapeptide tandem repeats (3).

Copper may contribute to PrPc conformation since the highly flexible amino-terminus of recombinant PrPc is more structured in the presence of copper (26). Moreover, spectroscopic data have suggested a bridged arrangement of coordinating histidine imidazole nitrogens binding four Cu^{2+} per PrPc molecule. This proposed coordination accounts for the cooperative binding of copper by PrPc (25).

Copper is an essential metal, which plays a fundamental role in the biochemistry of all aerobic organisms and is also required for the catalytic activity of several enzymes of interest to neurobiology (27). Free or incorrectly bound Cu^{2+} can catalyze the generation of damaging radicals such as hydroxyl radicals (28). Specific mechanisms have evolved in an appropriate compartmentalization and trafficking of this metal, avoiding oxidative stress (27).

Several groups have investigated the physiological meaning of the association between PrPc and copper. Brown and co-workers (24) reported that the copper content of membrane-enriched brain extracts from PrP^{-/-} mice is 10-15-fold lower than in wild-type controls while no significant difference was observed for other metals. These results suggested that PrPc is a major copper-binding protein in brain membrane fractions and controls the activity of other membrane-associated copper-binding proteins. The same group, using cerebellar cell cultures from mice expressing different levels of PrPc, demonstrated that cells with high levels of PrPc have an increasing resistance to oxidative stress compared to PrP^{-/-} cells (29,30).

The ability of PrPc to bind copper may modulate the activity of the major cellular antioxidant enzyme Cu/Zn superoxide dismutase (SOD-1) and consequently cellular resistance to oxidative stress. Western and Northern-blot analysis indicated that mice either lacking or overexpressing PrPc had levels of Cu/Zn SOD protein and mRNA equivalent to those expressed in wild-type mice. However, increasing levels of PrPc expression were linked to increased levels of Cu/Zn SOD activity (29,30). SOD-1 activity from cultured cerebellar neurons was approximately 50% the normal level in PrPc null mice and was elevated by 20% in transgenic mice overexpressing PrPc. In addition, experiments using cells metabolically labeled with radioactive copper have shown that Cu/Zn SOD immunoprecipitated from cells overexpressing PrPc has higher levels of radioactivity compared to PrPc-deficient cells (30). These observations suggest that PrPc may play some role in the delivery of copper to cuproenzymes such as SOD-1.

Morphological and biochemical investi-
gations of different PrPc-transgenic mouse lines provide strong evidence for a predominantly synaptic location of PrPc (31). A reduction in copper concentration in synaptic preparations of PrP<sup>−/−</sup> mice has been observed (24,31), indicating that PrPc is involved in synaptic copper homeostasis. In fact, electrophysiological studies have suggested that PrPc may regulate synaptic transmission by modulating copper content in the synaptic cleft (24,31).

More recently, a study using mass spectrometry methodology (32) failed to find any difference in the amount of ionic copper in subcellular fractions from brains of mice with different PrPc expression levels. They also showed that the enzymatic activity of SOD-1 and cytochrome c oxidase in brain extracts are similar for these groups, as also is the incorporation of copper into Cu/Zn SOD. The results of Waggoner et al. (32) differ from others (29,30) and suggest that PrPc is not the primary carrier responsible for copper entry into the brain and does not play a role in the specialized trafficking pathways involved in delivery of copper to SOD-1 in this tissue.

The exact mechanism by which copper and PrPc are functionally related is not known at present. PrPc, on the cell surface, may function as a sink for chelation of extracellular copper ions or as a carrier protein for uptake and delivery of these cations to intracellular targets. Pauly and Harris (10) have reported that copper stimulates endocytosis of PrPc from the cell surface via clathrin-coated pits. An attractive hypothesis to explain this observation is that PrPc may bind copper ions in the extracellular domain, deliver them to endocytic compartments and transfer these cations to other copper-carrier cytosolic proteins. The binding of copper could stimulate internalization of PrPc, altering its conformation and increasing its affinity for a putative endocytic transmembrane receptor (10).

Another possibility is that bound copper serves as an essential cofactor for an unknown enzymatic activity of PrPc. Indeed, it has recently been shown that recombinant chicken and mouse PrPc, as well as PrPc immunoprecipitated from mouse brain tissue, have SOD activity (33). These results suggest that PrPc has an enzymatic function dependent on copper incorporation and indicate that it could have a direct role in cellular resistance to oxidative stress.

Disturbances in copper homeostasis leading to CNS dysfunction are well documented in humans and animals. Some neurodegenerative diseases such as Menkes’ syndrome, Wilson’s disease, amyotrophic lateral sclerosis and Alzheimer’s disease are linked to altered copper transport and homeostasis (27). The evidence that PrPc has a role in copper metabolism may be important in understanding the pathogenesis of prion diseases, since loss of this copper-related function (as a result of conversion to PrPsc) could help to explain some features of these disorders. Interestingly, early studies have revealed that cuprizone, a copper-chelating agent, induces neuropathological changes in mice similar to those found in prion diseases (27), suggesting a role for copper in these disorders.

**PrPc-binding proteins**

The identification of PrPc-binding proteins can provide insights into the function of PrPc and the molecular mechanisms involved in prion diseases. There are a number of structural features within PrPc that might allow it to interact with other proteins. Two potential sites for binding are an amphipathic helix near the middle of the molecule that in other proteins has been implicated in protein-protein interaction and the GPI anchor which may internalize and deliver signals (34). Many studies have been conducted in recent years to probe the interaction of PrP with other molecules.

PrPc binds to a family of heparin-like
compounds; this interaction can influence the intracellular fate of the prion protein and inhibits the conversion of PrPc to PrPsc (35). Heparin is a sulfated polyanion closely related to cellular glycosaminoglycans, which in turn are associated with PrPsc in amyloid plaques. Therefore, it was proposed that these molecules act by directly competing with the binding of PrPc or PrPsc to cellular glycosaminoglycans. A recent publication characterized the sulfated polyanion-binding properties of recombinant PrPc protein using surface plasmon resonance and showed that the PrPc affinity for polyanions is parallel to their anti-scrapie formation potency (36).

PrPc also associates with PrPsc and this interaction is more efficient when the two isoforms have the same sequence, explaining the “species barrier” for prion transmission. Telling and co-workers (37) suggested that PrPc binds a species-specific macromolecule designated protein X which might function as a molecular chaperone in the formation of PrPsc.

Using a yeast two-hybrid system, Edenhofer and co-workers (38) identified the heat shock protein 60, a cellular chaperone, as a specific ligand for PrPc. The interaction site was mapped between amino acids 180 and 210 of the PrPc protein. In a recent report (39) BiP, another chaperone, was described to be associated with PrPc. This protein remains associated with PrPc mutants for an extended period of time. BiP normally interacts with misfolded or unassembled proteins, mediating their retrograde translocation for proteosomal degradation. The authors presumed that the impairment of the endosomal-lysosomal degradation leads to the accumulation of PrPsc.

Oesch and co-workers (34) also found that PrPc binds to glial fibrillary acidic protein (GFAP); however, studies with null mice for the GFAP gene revealed that this protein is not essential for TSE development (40).

In a study using a soluble tagged PrPc probe to screen an expression mouse brain cDNA library, six potential PrPc-binding proteins were identified (41). Four of them are coded by novel cDNAs, one is Nrf2 (NF-E2 related factor 2) and the last is apolipoprotein 1 (Aplp1), which plays a role in the pathogenesis of Alzheimer’s disease. The authors suggested that Aplp1 and PrP may possibly interact on the surface of neuronal cells or in the vicinity of the plasma membrane, but the role of this interaction in the development of prion or Alzheimer’s diseases remains to be clarified.

Bcl-2, an anti-apoptotic protein, was also reported to be associated with PrPc (42), with the binding site being located in the carboxy-terminal region of Bcl-2 which includes the transmembrane region. The authors suggested that Bcl-2 may act as a chaperone and induce conformational modifications in PrPc.

Another protein characterized as a PrPc ligand is the 37-kDa laminin receptor precursor. This protein interacts with PrPc in vitro and in vivo and is overexpressed in organs that accumulate PrPsc (43), suggesting that it could be a receptor or co-receptor for the prion protein in mammalian cells.

Our group has described the interaction of PrPc with a 66-kDa membrane protein both in vivo and in vitro, and antiserum against this ligand inhibits the toxicity of a prion-derived peptide towards neuronal cells in culture (21). The protein has been isolated on two-dimensional gels and its sequencing is underway. We will discuss the possible role of this protein in PrPc internalization and signaling in the last section of this article.

It is remarkable that, as previously described, PrPc binds to a large number of proteins; however, the physiological relevance of these interactions remains to be established.

We have recently characterized a specific high affinity binding between PrPc and laminin, an extracellular matrix protein.
Moreover, we provide for the first time consistent data regarding the physiological role of a PrPc association with another protein (44,45). These data will be discussed below.

**PrPc in neuronal survival and differentiation**

One of the proteins that associate with PrPc is laminin (44), a fact consistent with a possible PrPc function as a cell adhesion and recognition molecule. Laminin is an 800-kDa heterotrimeric glycoprotein consisting of two short and one long polypeptide chains, predominantly found in basement membranes and known to play a pivotal role in cell proliferation, differentiation, migration and death (46). The cellular responses triggered by laminin are mediated by its interaction with cell membrane receptors. The best known receptors are integrins but non-integrin laminin receptors have also been described (47).

In the CNS, laminin has likewise been shown to mediate neuronal differentiation through its interaction with integrins. This interaction is characterized by neurite formation and extension, migration of neurons both in vitro and in vivo (48), neuronal as well as axonal regeneration (48), and prevention of neuronal death after kainic acid injections (49). In the last model, tissue-type plasminogen activator was found to act by converting plasminogen into plasmin, which subsequently degrades laminin. Even though the cell receptor involved was not identified, it was clear that detachment of neurons from a laminin substrate was the determinant event for neuronal death, characteristic of seizure models.

Our group has observed that PrPc<sup>−/−</sup> mice are more sensitive to seizures caused by three convulsant agents including kainic acid, suggesting that the absence of PrPc renders animals more susceptible to neuronal death due to laminin degradation (16). Moreover, a recent study (50) using cells from PrPc<sup>−/−</sup> mice has shown that PrPc prevents serum deprivation-dependent apoptosis of neurons in culture and has suggested that PrPc might be similarly involved in neurite extension by these cells.

We have established the PrPc-laminin connection showing that PrPc is a specific, high affinity, saturable receptor for laminin and the binding site resides at the carboxy-terminal decapeptide (RNIAEIIKDI) of the laminin γ-1 chain (44). Indeed, neurite extension observed in primary cultures of hippocampal neurons in the presence of intact laminin-1 was quite sensitive to anti-PrPc antibodies, whereas that elicited by the carboxy-terminal peptide was completely inhibited by such antibodies. Furthermore, the neuritogenesis elicited by intact laminin was substantially decreased and was not inhibited by anti-PrPc antibodies when cells were derived from PrPc<sup>−/−</sup> mice, whereas no neuritogenesis could be elicited from such neurons by the carboxy-terminal peptide alone.

Very recently, we described the importance of PrPc-laminin interaction for neuronal cell adhesion. Indeed, using chromophore-assisted laser microscopy we confirmed the importance of this interaction for neurite extension and also showed its involvement in neurite maintenance (45).

The mapping of the decapeptide RNIAEIIKDI as the PrPc-binding site in the laminin molecule is particularly important since the γ-1 chain is the most conserved in all laminin types (48). Therefore, these data support the notion that PrPc-laminin interaction could be important in a variety of tissues in which both PrPc and different laminin isoforms are expressed.

In fact, PrPc is present on the surface of lymphocytes and its expression is increased when cells are activated by concanavalin A. Blockage of PrPc with specific antibodies suppresses mitogen-induced activation (51), suggesting that PrPc could participate in lymphocyte activation. On the other hand, laminin inhibits the proliferation of lymphocytes.
stimulated by concanavalin A (52). Whether the PrPc-laminin interaction participates in this event remains to be evaluated.

**PrPc and signal transduction**

Due to its cell membrane localization, PrPc could participate in cell signaling pathways and some progress has been made in the identification and characterization of the signaling involving PrPsc molecules.

It was described (53) that bradykinin-stimulated calcium responses in scrapie-infected cells was reduced by 30 to 50% compared with uninfected cells. The authors suggested that prion infection compromises calcium channel function.

After Forloni and co-workers (54) reported that a 21-amino acid fragment of the prion protein (PrP 106-126) could be toxic when chronically exposed to primary rat hippocampal cultures, some results about signal transduction were generated. This peptide forms ion-permeable channels in planar lipid bilayer membranes and these channels are freely permeable to the most common physiological ions like Ca$^{2+}$ and Na$^+$. Moreover, it was also observed that the neurotoxic peptide increases intracellular free calcium concentration in cultured microglia from wild-type and PrP$^{-/-}$ mice (56). Later, this peptide was found to be involved in the activation of tyrosine kinases Lyn and Syk, initiating a signaling cascade that results in a transient release of intracellular calcium and activation of classical protein kinase C and the calcium-sensitive tyrosine kinase PYK2. Activation of MAP kinases ERK-1 and ERK-2 follows as a subsequent downstream signaling event. An important point of the work of Combs et al. (57) is the demonstration that the signaling response elicited by neurotoxic peptide induces the production of neurotoxic products.

Nevertheless, important questions are still unanswered. How is the observed signal triggered? What is the nature of the signaling triggered by PrPc? What kind of molecules are involved in this event?

In an attempt to answer these questions our group has been working on the identification and characterization of a putative receptor for PrPc. We used the complementary hydropathy theory to predict a hypothetical peptide complementary to the human prion region from amino acid 114 to 126 (21) previously described to be neurotoxic in primary neuronal cultures (54) and responsible for PrPc internalization (58). Antiserum raised against the prion complementary peptide 114-126 recognized a 66-kDa membrane protein that binds PrPc both in vitro and in vivo. Furthermore, the complementary peptide as well as antiserum against it inhibited the toxicity of a prion-derived peptide towards neuronal cells in culture.

Shmerling and co-workers (59) reported in 1998 that mice with PrP lacking residues 32-121 or 32-134 but not those deleted from 32 to 106 presented severe ataxia and neuronal death limited to the granular layer of the cerebellum as early as 1-3 months after birth. Interestingly, the deleted PrPc region involved in the disease maps on the predicted binding site for the putative receptor described by us (21), suggesting that PrPc-receptor interaction should be important for the normal function of PrPc. It is tempting to speculate that the PrPc region from amino acids 106-126 contains the binding site for the receptor association and signal transduction. Since PrPc$^{-/-}$ animals do not develop the disease a PrP-like molecule might bind to the receptor and transduce similar signals. Moreover, the truncated PrPc proteins do not act as dominant negative molecules for the receptor since the defect was completely abolished by introducing one copy of a wild-type PrP gene (59).

Very recent data have shown that PrPc triggers cell signaling increasing the phosphorylation levels of the tyrosine kinase Fyn, and caveolin-1 was characterized as the intermediate factor between PrPc on the outer
membrane and the intracellular protein Fyn. However, the nature of the PrPc ligand responsible for the signal generation was not identified (60).

The two PrPc ligands characterized by us, the 66-kDa receptor (21) and laminin, seem to transduce signals through cAMP (Freitas AF, Martins VR and Brentani RR, unpublished results) and calcium (Lee KS, Prado MA, Brentani RR and Martins VR, unpublished results). Whether these two prion ligands can cooperate along the same pathway is still under investigation. Since laminin is located in the extracellular matrix and PrPc and PrPc 66-kDa receptor are located on the cell membrane, it is interesting to propose a connection between the extracellular milieu, the intracellular signaling, gene expression regulation and physiological events.

References


