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Physiology of the Prion Protein

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Linden R, Martins VR, Prado MAM, Cammarota M, Izquierdo I, Brentani RR. Physiology of the Prion Protein. *Physiol Rev* 88: 673–728, 2008; doi:10.1152/physrev.00007.2007.—Prion diseases are transmissible spongiform encephalopathies (TSEs), attributed to conformational conversion of the cellular prion protein (PrP^C) into an abnormal conformer that accumulates in the brain. Understanding the pathogenesis of TSEs requires the identification of functional properties of PrP^C. Here we examine the physiological functions of PrP^C at the systemic, cellular, and molecular level. Current data show that both the expression and the engagement of PrP^C with a variety of ligands modulate the following: *1*) functions of the nervous and immune systems, including memory and inflammatory reactions; *2*) cell proliferation, differentiation, and sensitivity to programmed cell death both in the nervous and immune systems, as well as in various cell lines; *3*) the activity of numerous signal transduction pathways, including cAMP/protein kinase A, mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt pathways, as well as soluble non-receptor tyrosine kinases; and *4*) trafficking of PrP^C both laterally among distinct plasma membrane domains, and along endocytic pathways, on top of continuous, rapid recycling. A unified view of these functional properties indicates that the prion protein is a dynamic cell surface platform for the assembly of signaling modules, based on which selective interactions with many ligands and transmembrane signaling pathways translate into wide-range consequences upon both physiology and behavior.

I. INTRODUCTION: A BRIEF ACCOUNT OF PRION PATHOLOGY

A. The Prion Diseases

Prion diseases correspond to anatomo-pathologically defined transmissible spongiform encephalopathies (TSEs) of an infectious, genetic, or sporadic nature and are characterized by neurodegeneration and protein aggregation. These diseases include kuru and Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE), also known as "mad cow disease," among others. The diseases can affect subjects at distinct age groups, course with a variety of motor or cognitive symptoms, and although their prevalence is relatively low, TSEs are still incurable and invariably fatal (262).

The pathogenesis of prion diseases is attributed to major changes in the metabolism of the cellular prion protein (PrP^C). Current understanding of TSEs evolved from the concept of the "prion," that is, a proteinaceous, nucleic acid-free, infectious particle (427).

B. The Prion Concept

Studies of kuru among the Fore people of Papua-New Guinea indicated that this spongiform encephalopathy was transmitted through ritual cannibalism. Indeed, injection of brain tissue from deceased patients into the brains of chimpanzees induced a similar disease, whereas the incidence of kuru sharply declined following the end of ritual cannibalism among the Fore. Scientists quickly noted the similarities among kuru, scrapie, as well as CJD (428).

The scrapie agent resisted doses of radiation that easily inactivated both viruses and bacteria (7), and the profile of sensitivity of the scrapie agent to various chemicals differed from both viruses and viroids, suggesting that the infectious agent might not depend on nucleic acids (24).

Further studies demonstrated that a protein unusually resistant to proteolysis was required for infectivity of diseased brain extracts (429), whereas no compelling evidence is available of the need for other components, especially nucleic acids. Therefore, although formal proof is still lacking, the prevailing hypothesis is that transmission of TSEs relies on a single protease-resistant protein component of the prion (427), which has received several denominations, such as PrP^{Sc}, for scrapie; PrP^{res}, due to its protease resistance; or PrP*, due to the uncertainties of whether the whole or only part of this component is actually pathogenic (5, 60).

C. Discovery of the Cellular Prion Protein

Sequencing of a microheterogeneous protein fraction of 27–30 kDa (PrP 27–30), produced by limited proteolysis of PrP^{Sc}, allowed the identification of a cellular gene (*Prnp*), that encodes a protein known as the cellular prion

protein (PrP^C), or simply prion protein (33, 429). Both the infective protein component of scrapie and PrP^C share the amino acid sequence and are encoded by a single-copy gene where the entire open reading frame is located in one exon (20, 396). This suggested that the distinctive properties of either the infective or the cellular prion proteins are determined by posttranslational modifications, and most research in the field was focused on the mechanisms by which PrP^C converts into PrP^{Sc}.

D. Conformational Conversion and Gain of Function in Prion Diseases

Following a brief flirt with the hypothesis that glycosylation imparts either normal or pathogenic properties to the prion protein (195, 503), structural studies of both PrP 27–30 and PrP^C led to the current view that PrP^{Sc} is an anomalous conformer of PrP^C, which results from the conversion of a predominantly α-helical structure into a predominantly β-sheet structure (18, 81, 169, 407). The latter explains the tendency of PrP^{Sc} to form compact, protease-resistant, protein aggregates, which in turn accumulate within the brain (452).

Although several models have been proposed to account for the formation of PrP^{Sc} aggregates, the basic proposal is that, following either infection with PrP^{Sc} or conversion of PrP^C into PrP^{Sc} associated with certain mutations thought to destabilize the protein (100, 221), binding of PrP^{Sc} to PrP^C leads to further conversion, thus resulting in accumulation of PrP^{Sc} at the expense of the normal PrP^C (Fig. 1). This hypothesis is consistent with the progressive nature of all variants of the prion diseases, as well as with the resistance of *Prnp* knockout mice to infection with scrapie brain extracts. It is also thought to underlie the predominant sporadic forms, in which pathogenesis might start with spontaneous conver-

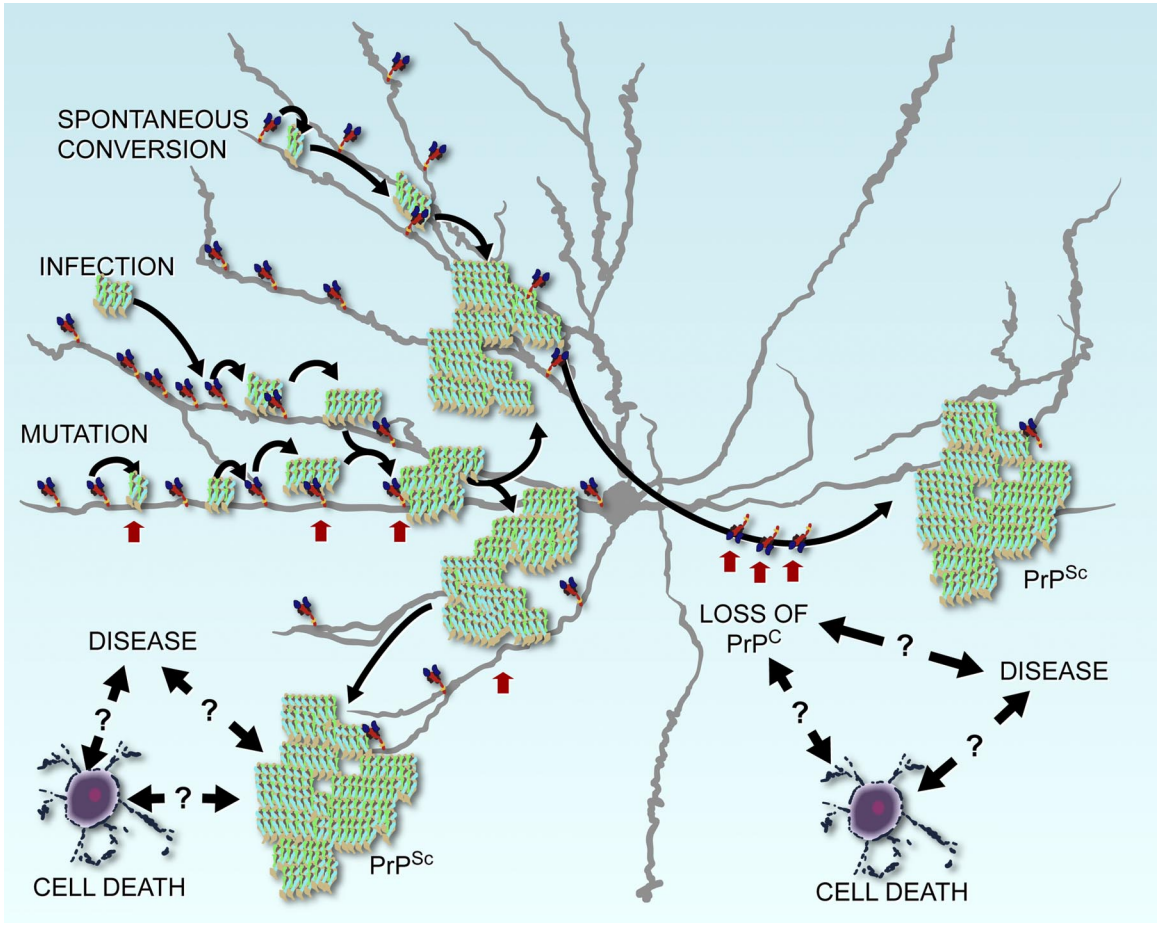

FIG. 1. The gain- and loss-of-function hypotheses of prion disease pathogenesis. According to prevailing views, a pathogenic conformer (in bright green) can enter the nervous system either through infection or as a result of conformational conversion of the normal protein (PrP^C, two-pronged fork shapes). The abnormal conformer coopts normal molecules into an aggregate formed by the predominantly beta sheet-containing abnormal protein (growing chunks of green elements), at the expense of the predominantly α-helical PrP^C (*left* to *right* in the figure). The basic tenet of the gain-of-function hypothesis is that PrP^{Sc} aggregation sensitizes neurons to programmed cell death and leads to prion disease. In turn, the loss-of-function hypothesis is based on the idea that loss of PrP^C leads to cell vulnerability and prion disease. The two hypotheses are not mutually exclusive, and the interdependence among PrP^{sc} accumulation, cell death, and disease is still unsolved.

sion of a fraction of PrP^C by hitherto unknown reasons (153, 174, 564).

It is believed that accumulation of PrP^{Sc} is the main pathogenic event leading to neurodegeneration. PrP^{Sc}, as well as the $PrP^C_{106–126}$ fragment ($PrP^C_{105–125}$ in mouse), known as the neurotoxic peptide, induce cell death both in vitro and in vivo, for which microglial activation and glial reactivity appear to be instrumental. These data are taken as evidence that prion diseases are gain-of-function consequences of the formation of PrP^{Sc} (109).

E. The Loss-of-Function Hypothesis

Despite compelling evidence for conformational conversion in the course of the diseases, it is still not clear what leads to the accumulation and cytotoxicity of the pathological conformer. For example, although it is widely assumed that accumulation of PrP^{Sc} causes neurodegeneration, systematic examination of the brains of deceased patients revealed no spatial correlation between apoptosis and deposits of PrP^{Sc} (96, 133, 185). Accumulated PrP^{Sc} within PrP^C-expressing tissue grafted into the brains of *Prnp*-knockout mice does not damage the neighboring PrP^C-null tissue (40), and progressive accumulation of PrP^{Sc} in glial cells around PrP^C-null neurons does not induce cell death in the knockout neurons, also arguing against a direct cytotoxic effect of PrP^{Sc} (326–328).

Thus the pervasive gain-of-toxic-function hypothesis is still unproven, and current models assume that PrP^{Sc} propagates at the expense of depletion of PrP^C (564), which warrants an examination of the hypothesis that loss of function of PrP^C (469), or of neurochemical systems associated with PrP^C, contributes to the pathogenesis of TSEs (Fig. 1). Critical appraisal of loss-of-function components in prion diseases is, nonetheless, hampered by the controversies surrounding the physiological functions of PrP^C.

F. What Function?

Full appreciation of the functional roles of PrP^C has likely been abashed by the striking lack of phenotype reported for the first *Prnp* knockout mouse (63). The contrast between the undisturbed phenotype and the spectacular change in the sensitivity to disease in these mice (63, 430, see below) greatly advanced the field of prion pathology, whereas physiological roles of PrP^C were largely neglected.

Nonetheless, data on functional consequences of interfering with PrP^C have accumulated over time, and the multiplicity of seemingly unrelated findings, as well as the nature of this particular protein, warrant an attempt at extracting and consolidating the major themes.

The purpose of this article is a comprehensive review of the evidence for physiological functions of PrP^C. This subject has, in recent years, been approached from molecular, cellular, and systems view points, each of which constrains the possible answers to the following question: What is(are) the major function(s) of the cellular prion protein?

We approach the subject by first discussing the structure, expression, regulation, molecular interactions, and subcellular trafficking of PrP^C. Then, functional roles detected in the nervous and immune systems, as well as in other organs, are discussed at the systems level. Finally, the evidence for functional roles of PrP^C is examined at the level of cellular physiology. The main hypothesis examined in the context of this review is that the prion protein is a dynamic cell surface platform for the assembly of signaling modules, based on which selective molecular interactions and transmembrane signaling translate into wide-range consequences upon both physiology and behavior.

II. STRUCTURAL AND MOLECULAR BIOLOGY OF THE PRION PROTEIN

A. Structure of PrP^C

Following the cleavage of a 22-amino acid (aa) signal peptide, most of mammalian PrP^C is exported to the cell surface as an *N*-glycosylated, glycosylphosphatidylinositol (GPI)-anchored protein of 208–209 aa. The tridimensional structure of the prion protein was determined by nuclear magnetic resonance at acid pH (71, 213, 307, 311, 444, 445, 590), and its major features are briefly discussed here.

PrP^C contains an NH_2-terminal flexible, random coil sequence of ~100 aa, and a COOH-terminal globular domain of about another 100 aa. The globular domain of human PrP^C is arranged in three α-helices corresponding to aa 144–154, 173–194, and 200–228, interspersed with an antiparallel β-pleated sheet formed by β-strands at residues 128–131 and 161–164. A single disulfide bond is found between cysteine residues 179 and 214. The NH_2-terminal flexible tail comprises approximately residues 23–124, and a short flexible COOH-terminal domain corresponds to residues 229–230 (590). The structure of the globular half of human PrP^C is identical to various other mammals, as expected from a high degree of sequence identity (307, 311). Notably, despite the low sequence identity between PrP^C in chicken, turtle, or frog, and the mammalian proteins, the major structural features of PrP^C are remarkably preserved in those nonmammalian species (71). In addition, both tertiary structure as well as the thermal stability of PrP^C isolated from the brain of healthy calf were indistinguishable from bacterially expressed, recombinant bovine PrP^C (213, 307).

Full-length PrP^C is found in non-, mono-, or diglycosylated forms, corresponding to the variable occupancy of residues Asn-181 and Asn-197 in human PrP^C and Asn-180 and Asn-196 in mice (195). A rather large variety of *N*-glycans were found attached to both full-length and truncated PrP^C (408, 457), which may be differentially distributed in various areas of the central nervous system (CNS) (27, 124).

The role of glycosylation was addressed both regarding the susceptibility to conformational conversion, as well as upon the diversity of TSEs (278). As for functional properties of PrP^C, less is known. The nearly identical tridimensional structures of both recombinant PrP^C and the glycosylated PrP^C isolated from calf brain indicate that neither the attached *N*-glycans nor most of the GPI anchor impart major structural features by themselves (213).

However, molecular dynamic simulations suggest that some attached *N*-glycans may modulate PrP^C stability (128, 139, 598), although experimental evidence is still lacking (213). Nevertheless, protein glycosylation affects other protein properties, such as intracellular traffic and ligand binding (299, 413, 504), the latter of which may be modulated both by subtle effects on protein structure as well as by steric hindrance (32, 70, 237, 243, 393, 513). Indeed, glycosylation reportedly affects the recognition of various species of PrP^C by monoclonal antibodies in both the brain and in other cells (27, 289), and differing responses to certain monoclonal antibodies were described for cells bearing distinctly glycosylated forms of PrP^C (365). Analogous selectivity may impinge on the binding of PrP^C physiological ligands.

In turn, experimental data suggest that membrane attachment through the GPI anchor (502), as well as other PrP^C-membrane interactions, may modulate the structure of the protein. An early study showed that PrP^C tends to interact with lipid vesicles containing acidic lipids, depending on pH. This interaction induced a slight, but consistent, change in the circular dichroism (CD) spectrum of the full-length protein, and a robust change in the CD spectrum of an NH_2-terminal fragment, upon binding to phosphatidylserine-containing liposomes, but not to vesicles composed of phosphatidylcholine alone (369). A similar change in CD spectrum was also found upon binding of a phospholipid-conjugated full-length PrP^C to raftlike membranes (see sect. III*A*) containing phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cerebrosides, and cholesterol, but not to lipid vesicles containing phosphatidylcholine and cholesterol only (137). Molecular dynamics simulations suggest that, although secondary structure is largely preserved, the NH_2-terminal domain of PrP^C can interact with membrane lipids and even traverse the surface of the membrane (128). A recent study employed another phospholipid-conjugated PrP^C and showed that, despite a large difference between the CD spectra of this form when compared with anchorless PrP^c, insertion into model lipid membranes restored the secondary structure of the protein, as shown by attenuated infrared spectroscopy (205). The data, therefore, indicate that the GPI-mediated anchoring of PrP^C into membranes has but little effect on secondary structure. It remains to be tested whether GPI-anchoring modulates other properties, such as biological activity, as shown for GPI-anchored fibroblast growth factor (263). Interestingly, anchorless PrP^C undergoes an unusual amyloid conversion into PrP^{res} and aggravates disease induced in mice expressing GPI-anchored PrP^C, but does not mediate clinical symptoms in infected mice lacking GPI-anchored PrP^C (91).

Analysis of the effect of pH on the structure of the prion protein may also be relevant to physiological functions of PrP^C. Both biophysical methods and selective antibody binding to exposed epitopes showed that the structure of PrP^C is sensitive to pH (6, 128, 212, 341, 519). This is particularly evident for the NH_2-terminal flexible domain, upon which some degree of structure, as well as changes in antibody binding were imparted by acid pH. This may be relevant both to the binding of PrP^C partners as well as to binding affinity, due to the continuous subcellular trafficking of PrP^C (see below). In addition, local pH modulation by a putative electrostatic potential formed by highly charged glycoconjugates at the cell surface (90, 158), including the glycosylated residues in PrP^C itself, may also contribute to the structure and biological functions of PrP^C in situ.

Thus further work on both the structure of PrP^C in situ as well as on the effects of local microenvironmental cues upon protein structure is likely to shed light on functional properties of the prion protein.

B. Structure and Regulation of the *Prnp* Gene

Whereas the structure and regulation of the *Prnp* gene is important for epidemiological, diagnostic and/or therapeutic considerations, the current review focuses on aspects that may be relevant for physiological functions of PrP^C.

The *Prnp* gene contains either three (in rat, mouse, bovine, sheep) or two exons (in hamster, humans, tamar wallaby), of which a single exon codes for PrP^C protein (95, 210, 425, 432, 459, 569). Control of *Prnp* gene expression has been attributed to sequences within the 5′-flanking region, within the first intron, and to 3′-untranslated sequences. The *Prnp* promoter did not display methylation either in nerve growth factor (NGF)-treated and untreated PC12 cells or in the rat brain (593).

No TATA box was identified linked to the *Prnp* gene, whereas a CCAAT element was found in most cases. Analysis of the 5′-flanking region indicated that strong

promoter activity required sequences within ~80–150 bp upstream of exon 1 (22, 231, 324, 460). Nonetheless, in bovine *Prnp*, the proximal promoter sequence between −88 and −30 within the 5′-flanking region required the simultaneous presence of a region of ~770 bp within intron 1 to drive reporter activity, indicating an interaction between promoter and intronic sequences (231). In the mouse *Prnp* gene, two equally strong promoter sequences upstream of both exon 1 and of exon 2, as well as a suppressor sequence within intron 1, were shown to control promoter activity in transfected neuroblastoma cells (22).

Four conserved motifs of as yet unknown significance were also detected upstream of the *Prnp* gene in mouse, human, and sheep (281, 569), but not in the tamar wallaby (425). In addition, several insertions, including retrotransposons, were described within the *Prnp* gene and its promoter sequences (281).

Differing from the *Prnp* ORF, the degree of homology of potential promoter sequences among various mammalian species is variable. For example, whereas bovine and sheep 5′-flanking regions share 89% homology, the bovine 5′-flanking sequence displays only 46–62% homology with the corresponding sequences from rodent and human *Prnp* (231).

Evidence for posttranscriptional control includes the finding, within the *Prnp* 3′-untranslated region, of conserved nuclear-specific and maturation-specific polyadenylation signal sequences (180, 425). More importantly, two distinct transcripts encoding the same *Prnp* ORF were found in sheep tissues, produced by alternative polyadenylation. Their relative levels differed in either brain or spleen, and also among ovine, caprine, and bovine tissues, and affected the level of synthesis of PrP^C. Still, no alternative polyadenylation was found in either human or mouse tissues (180). An additional significant finding of the latter study was the discrepant levels of translation of one of the alternative transcripts between sheep brain and neuroblastoma cells (180).

Prnp is often labeled as a housekeeping gene, based on the absence of a TATA box, presence of CpG islands, and the identification of several Sp1 binding sites (20, 22, 432, 460). However, evidence that transcription of *Prnp* is modulated by chromatin structure (68), as well as the potential binding sites for many transcription factors, indicate that *Prnp* expression likely depends on a variety of cellular factors. Notwithstanding some species variation, the following elements were reported, in addition to Sp1, both in the 5′-flanking region and within the first intron: AP1, AP2, MZF-1, MEF2, MyT1, Oct-1, NFAT, POZ (BCL6); RP58 (ZNF238); NEUROG1; EGR4, Oct-1/Oct-2, NF-IL6, MyoD, p53, HSE, MRE, MLS, but not CRE, NF-κB or OTF-I (231, 324, 425, 460, 470, 490, 541, 569). Polymorphism of the bovine promoter modulated the expression of the *Prnp* gene, depending on interaction of transcription factors RP58 and Sp1 (470). It was, however, claimed that activity of a promoter sequence including exon 1 and upstream was independent on the level of Sp1 found in several transfected cell lines (515).

Expression of both PrP^C mRNA and protein are developmentally regulated, increasing postnatally with distinct time courses for various regions of the hamster, rat, and mouse brains (280, 293, 330, 349, 355). In addition, induction of *Prnp* mRNA expression in the mouse was first detected between embryonic days 8.5 and 9, coinciding with the transition from anaerobic to aerobic metabolism (355).

Consistent with developmental regulation, injections of NGF into the brains of neonatal hamsters upregulated *Prnp* mRNA together with activity of choline acetyltransferase in regions that contain NGF-responsive cholinergic neurons (364). Neuronal-like differentiation of PC12 cells induced by either interleukin (IL)-6 or NGF was accompanied by increased expression of *Prnp* mRNA (279). The induction of *Prnp* by NGF was shown to depend on opposing effects of either the mitogen-activated protein (MAP) kinase or the PI 3-kinase/Akt pathways and occurred in fibroblasts without simultaneous differentiation, indicating that the effects on *Prnp* are not a trivial consequence of the NGF-induced differentiation (593). Expression of *Prnp* mRNA and PrP^C protein also showed signs of differential regulation along two diverging differentiation pathways of a neuroectodermal cell line (375).

Treatment with cytokines of various neural-like cell lines, as well as teratocarcinoma-derived differentiated neurons, also indicated that distinct cell types respond differently to growth factors. Thus SK-N-SH neuroblastoma cells upregulated *Prnp* mRNA in response to IL-1β, tumor necrosis factor (TNF)-α, and phorbol ester and downregulated *Prnp* mRNA upon treatment with interferon (IFN)-γ. In contrast, no response to these cytokines was detected in IMR-32 neuroblastoma, U-373MG astrocytoma, or teratocarcinoma-derived differentiated neurons (474).

In contrast to neuronal-like differentiation in vitro, the mRNA for both glial fibrillary acidic protein (GFAP) and PrP^C in cultured astrocytes correlated during proliferation, but, contrary to GFAP, *Prnp* transcription did not respond to several growth factors (78, 279). Therefore, increased levels of expression of *Prnp* correlate with brain development and neuronal differentiation, but apparently not with astroglial differentiation.

Still related with development and tissue differentiation, *Prnp* mRNA in cell cultures of perodontal ligament (PDL), a neural crest-derived connective tissue, was upregulated in response to MRP8 and downregulated by platelet-derived growth factor (PDGF), both of which are chemotactic for PDL cells (261). Differentiation-related regulation of *Prnp* expression was also reported in pan-

creatic insulin-producing beta cells, following treatment with growth hormone and dexamethasone (16).

Stress modulates the expression of *Prnp*. In neuroblastoma cells, heat shock increased both PrP^C mRNA and protein (491), together with Hsp70 (490). Both hyperbaric oxygen and hypoglycemia upregulated the *Prnp*, 70-kDa heat shock protein (Hsp70), and JNK genes, and the activation of the *Prnp* promoter depended on the presence of one of the two heat shock elements identified in its sequence (489, 493), similar to neuroblastoma cells subject to hypoxia-reoxygenation (492). Although the activation of HSE may be consequent to phosphorylation of HSF1 by either JNK or Erk under hyberbaric oxygen or hypoxia-reoxygenation paradigms, respectively (490, 492), direct evidence for this particular chain of events is lacking.

Also related to stress, inflammation both in skin and in gastrointestinal epithelium led to upregulation of PrP^C (405, 406). Altered redox states correlated with the content of PrP^C in prostate tumor spheroids, possibly related to a reactive role of PrP^C to oxidative stress (477; see sect. II*E*). Nonetheless, *Prnp* mRNA was not quantified; thus it is unclear whether any of these conditions actually induces increased gene expression or, alternatively, prevents PrP^C degradation.

Chronic copper overload resulted in upregulation of *Prnp* in two lines of fibroblasts from mutant mice which accumulate abnormally high levels of copper in normal culture medium (10). Hippocampal and cortical neurons in culture upregulated *Prnp* transcription in response to copper, and a reporter vector driven by the *Prnp* promoter was activated by copper when transfected into PC12 cells, but not into C6 glioma cells. Responses both to copper and to cadmium depend on the presence of a putative metal responsive element (MLS1), although transcription factor MTF-1 is not involved (541).

Expression of *Prnp* is also regulated among immune cells. Both PrP^C mRNA and protein were downregulated upon differentiation of bone marrow cells along the granulocyte lineage, an effect also found following retinoic acid-induced granulocyte differentiation of the HL60 leukemia cell line (132). More recently, it was shown that the effects of retinoic acid on PrP^C mRNA and protein depend on protein synthesis, but could be dissociated from the retinoic acid-induced differentiation of the cell line (458).

Apart from direct evidence such as that obtained for heat shock or metal responsive elements (493, 541), the roles of most of the potential regulatory elements identified in *Prnp* are still unknown. Transduction of regulatory signals generated by phorbol ester or certain growth factors and cytokines upon immune or neural cells (135, 404, 474) may be mediated by AP-1, NF-IL6, and NFAT elements (324, 425). Proliferation or differentiation signals may relate, for example, with MZF-1, MyT1, and MyoD binding sites, while tissue- and cell-specific expression (78, 180, 279, 474) may depend on elements such as MEF2 or Oct-1 (425). Little data are available to identify the chain of events linking most cellular responses with *Prnp* expression. Understanding of PrP^C function, particularly in signal transduction, will require further functional analysis of the *Prnp* promoter.

Several studies reported altered expression or surface exposure of PrP^C without examination of *Prnp* mRNA levels (135, 289, 315, 404). However, a significant C1q-dependent upregulation of PrP^C protein was induced in follicular dendritic cells of spleen germinal centers, by intravenous immunization of mice with either preformed immune complexes or vesicular stomatitis virus. In this case there was no change in mRNA content, indicating that the PrP^C was modulated posttranscriptionally (308).

C. Expression and Distribution of PrP^C

The prion protein is highly expressed within the nervous system, although its content varies among distinct brain regions, among differing cell types, and among neurons with distinct neurochemical phenotypes. Various cellular components of the immune system, in the bone marrow, blood, and peripheral tissues, also express substantial amounts of PrP^C. Finally many other organs and tissues present PrP^C expression (Table 1).

Notwithstanding, several discrepancies can be noted among published reports. Thus PrP^C was demonstrated in neuronal cell bodies, contrary to an exclusively synaptic localization suggested in certain studies. As for the latter, conflicting results were reported as to whether PrP^C resides in either or both pre- and postsynaptic components. Further discrepancies surround glial expression of PrP^C (Table 1). Multiple reasons may account for these controversial findings, such as the use of various antibodies. Although the latter usually cross-react among mammalian PrP^C, it is possible that low protein levels in certain cell types may be missed due to either subtle differences in protein structure or glycosylation patterns (27, 124). Furthermore, many studies were done with the use of a single monoclonal antibody, which may introduce bias due to variable degrees of epitope exposure among distinct cell types (27).

Although mRNA and protein levels usually correlate well, one particular study revealed marked disparities between PrP mRNA, shown by in situ hybridization, and PrP^C protein, revealed by immunohistochemistry (150). Here, PrP^C-negative dopaminergic neurons of the substantia nigra, olfactory glomeruli, and locus coeruleus displayed a high PrP mRNA content. Also, within the deep cerebellar nuclei, all neurons expressed high levels of PrP mRNA, but only interneurons contained high levels of PrP^C (150).

The reason for this disparity between mRNA and protein levels of PrP^C within the CNS is not known.

TABLE 1. *Expression and distribution of prion protein*

Species	Organ/Tissue	Cell Type/Subcellular Distribution	Technique	Detection Level and Regulation	Reference Nos.
Hamster	Brain	Neuron cell bodies	IHC, WB		123
Mouse, hamster	Brain	Neurons, intracellular	IHC		420
Hamster, macaque, human	Brain	Presynaptic, not in cell bodies	WB, IHC (mAb 3F4), EM		468
Hamster	Developing brain	Mainly along axon tracts	IHC (mAb 3F4)	Developmentally regulated, remains high in adult olfactory bulb and hippocampus	467
Mouse	Embryonic brain, spinal cord, PNS	Neurons, nonneuronal cells	ISH	Developmentally regulated	330
Mouse	Brain	Neurons, neuronal processes, not in glia	IHC (pAbs GAx), ISH	Varied both among and within brain regions, depending on cell type and neurochemical phenotype. Protein often not correlated with mRNA	150
Hamster, human	Hippocampus	Presynaptic	IHC, EM		156
Hamster	Cerebellum	Pre- and postsynaptic	IHC, EM		191
Human	Cerebellum	Neurons	IHC (mAb 3F4)	Low level in cerebellar granule cells of normal brain	143
Rat	Cerebellum	Neuron and glial cell bodies and processes	IHC (mAb 8H4), EM		275
Rat	Neonatal retina	Retinal precursors and differentiating neurons	WB, IHC (mAb 6H4, pAb N10, pAbMo)		92
Hamster	Brain (hippocampus, septum, caudate nucleus, thalamus), DRG, blood, heart, skeletal muscle, lung, gut, spleen, testis, ovary, and others	Both in neuronal cell bodies and neuropil	WB, IHC (mAb 3F4 + 2 distinct pAbs), EM	Highest in hippocampus	25, 157
Mouse	Olfactory bulb, PNS, bone marrow, lymphoreticular system, gut, lung, kidney, testis, skin, not liver	Peripheral axons, neuron cell bodies, not glia; hemopoietic progenitors, megakaryocytes, monocytes, not granulocytes in bone marrow; dendritic cells, pericytes, intraepithelial lymphocytes in various tissues	IHC (pAbs GAx, mAb SAF61), ISH	Varied both among and within distinct organs and tissues. Scattered cells with high expression. Protein correlated with mRNA	151
Mouse	Intestine	Submucosa, muscularis mucosa	ISH		330
Hamster	Stomach, intestine, lung, kidney	Secretory globules	IHC (pAbs Br-1, R073, P38, mAb 3F4), EM		155
Human	Stomach, kidney, spleen	Secretory globules	IHC (pAbs Br-1, R073, P38, mAb 3F4), EM		155
Bovine	Ovary	Ovarian follicles	Microarray, real-time PCR, WB (mAb HumP)	Upregulated in theca cells of dominant, as compared with subordinate follicles	152
Sheep	Spleen, lymph node, lung, heart, kidney, skeletal muscle, uterus, adrenal gland, parotid gland, intestine, mammary gland, not liver, not pancreas		WB (homemade pAbs and mAbs), NB		211
Mouse, hamster, human	Muscle	Subsynaptic sarcoplasm, not postsynaptic plasma membrane	IHC (pAb R254, R073), EM		175
Mouse	Muscle	Myoblast cell lines	WB (pAb Ra5)	Upregulated with differentiation of myotubes from myoblasts	53

TABLE 1.—*Continued*

Species	Organ/Tissue	Cell Type/Subcellular Distribution	Technique	Detection Level and Regulation	Reference Nos.
Human	Blood	Lymphocytes and lymphoid cell lines, monocytes, not erythrocytes, not mature granulocytes	FC (mAb 3F4), NB	Downregulated with differentiation in granulocytes	77, 132
Human	Blood	monocytes, T cells, NK cells, B cells	FC (mAbs 3F4, 3F5)	Upregulated in activated T cells and monocytes, not uniform among NK cells, low in B cells	135
Human	Blood	monocytes, T cells, B cells, DC	FC (4 distinct mAbs)	Upregulated in activated T cells	289
Human	Blood	$CD34^+$ cells, megakaryocytes, platelets	WB, FC, IHC (mAb 6H4), EM (pAb P3), RT-PCR	Surface PrP^C increases upon platelet activation	505
Mouse	Bone marrow, thymus, fetal liver, not spleen, not peritoneum	Hemopoietic stem cells, immature thymocytes, not peripheral blood leukocytes, not gut intraepithelial lymphocytes	FC (mAb 6H4)		270
Mouse	Skin, thymus, spleen, lymph nodes	DC	FC (mAb SAF83)	Heterogeneous distribution among DC subtypes; absent in B220+ DCs; upregulated with DC maturation	337
Bovine	Spleen, lymph nodes, blood	Follicular DC, B cells, lymphocytes, monocytes, PMN	IHC, FC (mAb 6H4)	Monocytes and PMN immunoreactivity low, B cells high	403
Sheep	Blood	B cells, T cells, monocytes, not granulocytes, not erythrocytes	FC (mAbs 8H4, 5B2, 7A12), RT-PCR	Platelet fraction contained PrP mRNA, not surface PrP^C	203
Sheep	Blood	PBMC, platelets, not granulocytes	WB, FC (mAbs FH11, 4F2. 8G8, 6H4)	PrP^C in platelets intracellular only; level of PrP^C expression in B cells higher in scrapie-susceptible than in scrapie-resistant genotype	193

DC, dendritic cell; EM, electron microscopy; FC, flow cytometry; GAx, glutaraldehyde cross-linked epitopes; IHC, immunohistochemistry; ISH, in situ hybridization; mAb, monoclonal antibody; NB, Northern blot; pAb, polyclonal antiserum; pAbMo, polyclonal antiserum raised in PrP-null mouse; PMN, polymorphonuclear; WB, Western blot; 3F4, 3F5, 4F2, 5B2, 6H4, 7A12, 8G8, 8H4, FH11, HumP, SAF61, SAF83, designations of monoclonal antibodies; Br-1, N10, p3, P38, Ra5, R254, R073, designations of polyclonal antisera.

Notably, in a simultaneous study from the same group, employing the same techniques, mRNA and protein correlated well in both peripheral neurons and nonnervous tissues (151). The authors suggested that an exceptionally fast turnover of PrP^C in cerebellar granule cells may explain the disparity. This, however, does not fit studies of PrP^C turnover in vitro, because primary cultures of granule cells and of splenocytes showed similar half-lives for PrP^C (410), whereas there was no disparity between mRNA and protein levels in the spleen (151). The in vitro turnover study (410) also indicated that primary culture cells degraded PrP^C more rapidly than cell lines, but overexpressing transgenic tissue was employed in this investigation. Therefore, further studies of the turnover of PrP^C in distinct neuronal populations, as well as other cell types, are warranted.

The studies of the mouse brain and peripheral nervous system (150, 151) employed a range of antibodies raised against epitopes along the whole PrP^C molecule, coupled to carrier by disuccimidyl-glutarate, which cross-links free amide groups in a reaction similar to that of glutaraldehyde. The authors aimed at detecting PrP^C cross-linked to its normal positions at the cell surface, which is deemed to occur in glutaraldehyde-fixed tissue (150). This procedure highlights the additional caveat, that fixation and handling may bias the detection of protein at the surface of expressing cells due to the continuous trafficking and turnover of PrP^C (see sect. III).

Reports in the immune system also harbor some controversy. Thus a study of mouse lymphoid tissue employing flow cytometry with the 6H4 monoclonal antibody (270) differed from data obtained by immunohistochem-

istry (151) as to the presence of PrP^C in spleen cells, peritoneal cells, peripheral blood leukocytes, and intestinal intraepithelial lymphocytes, suggesting that maturing mouse immunocytes either downregulate PrP^C to a level undetectable by flow cytometry or, alternatively, mask the 6H4 epitope through posttranslational changes in PrP^C. On the other hand, a study of bovine lymphoid tissue also by flow cytometry with the 6H4 antibody confirmed the negative results in mouse for a variety of T cells and myeloid cells, but PrP^C was detected in lymphocytes and, to a lesser extent, in monocytes and even polymorphonuclear cells (403). It is not clear whether and which of these distinct findings are related to species differences, degree of surface exposure of PrP^C, or technical artifacts.

An important additional finding is that the expression of PrP^C depends on both maturation and cellular activation in various cell types. Thus PrP^C is developmentally regulated in both the differentiating brain and muscle (53, 330) and in maturing granulocytes (77, 132). Also, PrP^C is upregulated by activation of T cells, monocytes, and dendritic cells (135, 289, 337), and intracellular PrP^C migrates to the cell surface upon activation of platelets (193, 203, 505). Therefore, heterogeneous expression of PrP^C among various cell types, organs, and species may be additionally imparted by conditions such as the raising of laboratory mice in specific pathogen free (SPF) conditions and differential exposure of distinct animal cohorts to inflammatory/stress inducers.

The overall evidence for heterogeneous, cell type-specific, and regulated expression of PrP^C is relevant both for the understanding of pathogenesis as well as for the interpretation of physiological functions of PrP^C. It should be carefully taken into account when examining transgenic and PrP-null mice, in which the expression of PrP^C is evenly modulated in all organs and tissues, as well as in cases when multiple cell types and systems may be involved, such as complex behavior mediated by interacting neural pathways and diverse neurochemical systems, or the cross-talk of inflammatory and neurodegenerative effects.

D. PrP^C Ligands

Many reports are available on putative ligands of the prion protein, most of which aimed at the identification of the elusive "protein X," believed to be a necessary component of the pathogenic conformational conversion (427, 528). Findings have been reviewed, usually grouping ligands of PrP^C together with ligands of PrP^{Sc} (282, 333). Table 2 shortlists data related to candidate physiological ligands of PrP^C.

BLAST searches performed while writing this review have provided additional clues as to the identity of some previously published, unidentified hits. For example, Prion interactor 1 (Pint1), which was reportedly homologous to several ESTs (500), contains a conserved motif found in exonuclease families of proteins (Fig. 2*A*). These data, together with evidence of interaction of PrP^C with other nuclear proteins, as well as with nucleic acids (114, 163, 511, 586), are consistent with functions of PrP^C within the nucleus, the nature of which remains obscure.

We also found that a previously unidentified clone 3c2 from a *lambda-gt11* mouse brain cDNA library (586) has a high degree of homology with a G protein-coupled receptor (GPCR)-associated sorting protein (GASP), whereas clone 12b2, previously equated with Fbx6 (587), is, indeed, homologous to a conserved domain found in F-box proteins (Fig. 2, *B* and *C*).

Both the GASP and the Fbx protein families are related to mechanisms of protein trafficking and turnover. The GASP family comprises several proteins, many of which are predominantly expressed in the CNS and associate with COOH-terminal cytoplasmic tails of various GPCRs, including glutamate, acetylcholine, and neuropeptide receptors (204, 494). The founding member of the family is required for the choice between recycling and degradation of the δ-opioid receptor (573). It is, nonetheless, unclear whether the topology of these proteins may allow control over the traffic of PrP^C.

F-box-only proteins are responsible for substrate recognition by the SCF complex, a major type of E3 ubiquitin ligase, and therefore regulate protein stability and degradation (206). The highest degree of homology in our BLAST search of clone 12b2 was found with the F-box-only protein Fbxo2 (Fig. 2*C*), which binds specifically to *N*-linked high-mannose oligosaccharides and is associated with the ubiquitination of *N*-glycosylated proteins retrotranslocated from the endoplasmic reticulum to the cytosol by the quality control mechanisms (588). Interestingly, the high-mannose, immature form of PrP^C that precedes complex glycosylation appears to be the most sensitive to conformational conversion into PrP^{Sc} (580), and therefore, turnover mechanisms dependent on the binding of PrP^C to Fbxo2 may be key components of prion disease pathology.

Binding domains along the whole extent of the PrP^C molecule have been identified for a number of ligands (Fig. 3). It should, however, be noted that the techniques used for those studies were quite variable, and many interactions detected by screening methods have yet to be confirmed by biochemical and cell biological approaches. In particular, some putative ligands appear not to be accessible from the usual topology of PrP^C, which constitutes a critical question to be addressed by future studies.

Notwithstanding, for a few PrP^C ligands, data are accumulating on cellular responses upon their binding to the prion protein. These ligands are described in some

TABLE 2. *Putative physiological ligands of the prion protein*

Ligand	Description	Method	Reference Nos.
Synapsin Ib	Synaptic vesicle release regulator	Two-hybrid screen	500
PSD-95	Postsynaptic density scaffolding protein	lambda-gt11 mouse brain cDNA library	587
Grb	Adaptor protein for tyrosine kinase receptors	Two-hybrid screen	500
GASP	G protein-coupled receptor-associated sorting protein (Fig. 2*B*)	lambda-gt11 mouse brain cDNA library	586; this study
14-3-3	Intracellular scaffolding protein	Overlay, MS	475
CK2	Protein kinase	Overlay, SPR	354
Fyn, ZAP-70	Protein tyrosine kinases, soluble	coIP	343
PTPD1	Protein tyrosine phosphatase, soluble	lambda-gt11 mouse brain cDNA library	587
Aldolase C/zebrin II	Glycolytic pathway enzyme	Overlay, coIP, MS	511
Bcl-2	Anti-apoptotic protein	Two-hybrid screen	271, 272
Fbx6/Fbxo2	Substrate recognition unit of ubiquitin ligase complex (Fig. 2*C*)	lambda-gt11 mouse brain cDNA library	587; this study
GFAP	Intermediate filament protein	Overlay	395
DNA	Nucleic acid	Biophysical measurements	114
RNA	Nucleic acid	EMSA	163
hnRNP A2/B1	RNA-binding protein	Overlay, coIP, MS	511
Nrf2	Transcription factor	lambda-gt11 mouse brain cDNA library	586
Pint1	Exonuclease motif (Fig. 2*A*)	Two-hybrid screen	500; this study
Hsp60	Chaperone	Two-hybrid screen	138
αB-crystallin	Stress-induced small hsp	Two-hybrid screen, N-PAGE, optical biosensor	514
BiP/Grp78	Endoplasmic reticulum chaperone	coIP	242
Hop/STI1	Co-chaperone	Complementary hydropathy, binding assays	338, 591
Laminin	Extracellular matrix component	Binding assay	103, 183
LRP/LR	Laminin receptor precursor/laminin receptor	Two-hybrid, cell binding	171, 226, 442
NCAM	Cell adhesion molecule	Cross-linking, coIP	471, 479
LRP1	Scavenger receptor	Cross-linking, coIP, binding assay	525; R. J. Morris, personal communication
β-Dystroglycan	Transmembrane protein	coIP, detergent sensitivity	256, 257
APLP1	Amyloid precursor-like protein	lambda-gt11 mouse brain cDNA library	586
Heparin/heparan sulfate	Glycosaminoglycans	SPR, ELISA	216, 408, 557
50, 56, 64, 72, 110 kDa proteins	Unidentified	Overlay	395

MS, mass spectrometry; SPR, surface plasmon resonance; EMSA, electrophoretic mobility shift assay; coIP, coimmunoprecipitation; N-PAGE, nondenaturing polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

detail below, and their physiological relevance is discussed in later sections.

With the use of a yeast two-hybrid approach, followed by cell-binding experiments, it was shown that PrP^C binds the 37-kDa laminin receptor precursor (LRP), and/or the 67-kDa laminin receptor (LR), both of which have been identified as receptors for distinct viruses. Two binding sites each in both PrP^C and LRP were identified. One set of binding sites (hamster $PrP^C_{144–179}$; $LRP_{161–179}$) provided direct interaction, and another ($PrP^C_{53–93}$; LRP undetermined, either between aa 101–160 or 180–295) underlies heparan sulfate proteoglycan (HSPG)-mediated interaction (171, 226, 443). PrP^C binds both heparin and heparan sulfate through at least three domains, one of which coincides with the HSPG-dependent LRP-binding site $PrP^C_{53–93}$ (216, 408, 557). Importantly, all interactions of PrP^C with LRP/LR were independent of either the laminin-specific integrin VLA-6 or galectin-3 (171, 226). In turn, $LRP_{161–179}$ binds both PrP^C and laminin (443), and $PrP^C_{173–182}$ binds laminin itself (103), at the sequence RNIAEIIKDI (known as the LN γ-1 peptide) of the conserved COOH-terminal laminin γ chain, with functional consequences (183, see sects. IV*G* and VII*B*).

In turn, in situ cross-linking of N2a cells in the presence of low concentrations of formaldehyde resulted in a high-molecular-weight complex composed of PrP^C coupled to various isoforms of neural cell adhesion molecule (NCAM). The binding site in NCAMs was found in β-strands C and C′ within the two consecutive fibronectin type III domains, whereas the binding site in PrP^C was located in the NH_2 terminus, helix A (residues 144–154) and the adjacent loop region of PrP^C (479). PrP^C and NCAM also coimmunoprecipitated from brain tissue (471).

A distinct approach led to the discovery of a strong interaction between PrP^C and the cochaperone Hsp70/Hsp90 organizing protein/Stress-induced protein 1 (hop/STI1). Complementary hydropathy theory (42, 264) led to the design of a putative PrP^C-ligand peptide which proved to actually bind PrP^C. An antibody raised against the designed peptide recognized a single PrP^C-binding protein in brain extracts (338), identified as

A

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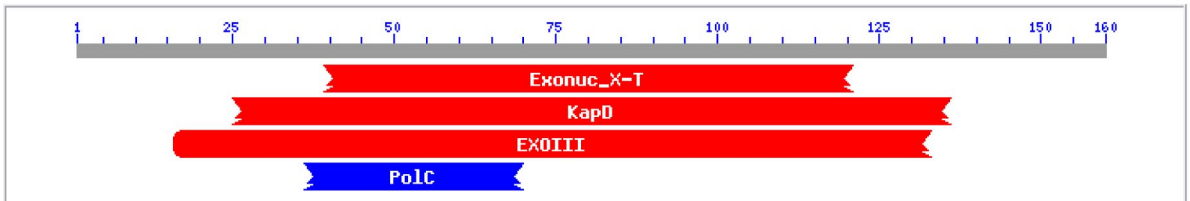
Pint 1 MEYPYDVPDYAHMAMEASEFHPGGHRYGIRIRGVDHPQLTPFCTELTGIIQAMVDGQPSLQQVLERVDEWMAKEGLLDPN
       VKSIFVTCGDWDLKVMLPGQCQYLGLPVADYFKQWINDKKVPLSSSQGLWFSCPSPPPPTLVSFSFFSQYSPFSSLGNLY
```

Options: **Database:** cdd.v2.10 **Low complexity filter:** yes **E-value threshold:** 0.010 **Max. hits:** 500
Data Source: Live blast search **RID** = 1167738431-24163-101727200392.BLASTQ3
System: **Search creator:** newblast **Software:** blastp 2.2.15 Sep-24-2006 **Service:** rpsblast



Descriptions

Title	Pssmid	Multi-Dom	E-value
[+]pfam00929, Exonuc_X-T, Exonuclease. This family includes a variety of exonuclease prot...	41004	No	5e-11
[+]COG5018, KapD, Inhibitor of the KinA pathway to sporulation, predicted exonuclease [Ge...	34623	No	3e-10
[+]smart00479, EXOIII, exonuclease domain in DNA-polymerase alpha and epsilon chain, ribo...	47785	No	8e-10
[+]COG2176, PolC, DNA polymerase III, alpha subunit (gram-positive type) [DNA replication...	32359	Yes	0.00007

B

```
3c2          CDAEPGAEEGAIGGSLFWTEEKPSLGAVARDEVRPESEEEALFGSWFWDRDEACFDPNPTPVYTAKSRYRDPEEDLNLASRPKTWDEVTIEF
             C++EPG EEGAIGGS +W EEK SLGAVAR+E +PESEEEA+FGSWFWDRDEACFD NP PVY    R+RD  E+LN +SRP+TWDEVT+EF
NP_612446    CESEPGTEEGAIGGSAYWAEEKSSLGAVAREEAKPESEEEAIFGSWFWDRDEACFDLNPCPVYKVSDRFRDAAEELNASSRPQTWDEVTVEF
            407                                                                                        498
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C

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12b2         RRNLLRNPCGEEDLEGWSDVEHGGDGWKVEELPGDNGVEFTQDDSVKKYFASSFEWCRKAQVIDLQAEGYWEELLDTTQPAIVVKDWY
             RRNLLRNPCGEEDLEGW DVEHGGDGW+VEELPGD+GVEFT D+SVKKYFASSFEWCRKAQVIDLQAEGYWEELLDTTQPAIVVKDWY
AAF04515     RRNLLRNPCGEEDLEGWCDVEHGGDGWRVEELPGDSGVEFTHDESVKKYFASSFEWCRKAQVIDLQAEGYWEELLDTTQPAIVVKDWY
             81

12b2         SGRTDAGSLYELTVRLLSENEDVLAEFATGQVAVPED---GSWMEISHTFIDYGPGVRFVRFEHGGQDSVYWKGWFGARVTNSSVWVEP
             SGR+DAG LYELTV+LLSE+E+VLAEF++GQVAVP+D   G WMEISHTF DYGPGVRFVRFEHGGQ SVYWKGWFGARVTNSSVWVEP
AAF04515     SGRSDAGCLYELTVKLLSEHENVLAEFSSGQVAVPQDSDGGGWMEISHTFTDYGPGVRFVRFEHGGQGSVYWKGWFGARVTNSSVWVEP
                                                                                                    257
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FIG. 2. Identification of PrP^C ligands from previously published orphan sequences. BLAST search shows that the Pint1 (Prion interacting protein 1), identified in a two-hybrid mouse screen, contains a conserved domain found in a variety of exonucleases (*A*). Two clones previously detected in a lambda-gt11 mouse brain library correspond to a G protein receptor-associated sorting protein (clone 3c2, *B*) and to a conserved domain found in F-box proteins (clone 12b2, *C*). The sequences shown are for human GASP (*B*) and human Foxb2 (*C*). Sequence homology is highlighted (bold characters). Clone sequences in *A* from Spielhaupter and Schatzl (500) and in *B* and *C* from Yehiely et al. (586).

hop/STI-1 (591). Hop/STI-1 is an adaptor and modulator of the activities of Hsp70 and Hsp90 and may have additional roles in cell metabolism (394, 589 for reviews).

Although heat shock proteins and cochaperones are mainly intracellular, hop/STI-1 has also been located extracellularly. Thus hop/STI-1 is secreted by certain tumor cell lines, together with several chaperones (141), as well as by primary astrocyte cultures (F. R. S. Lima, C. P. Arantes, A. G. Muras, R. Nomizo, R. R. Brentani, and V. R. Martins, unpublished results). These data are consistent with a physiological interaction of hop/STI1 with PrP^C at the cell surface.

Finally, recent studies have implicated the low-density lipoprotein receptor-related protein 1 (LRP1) with endocytic trafficking of PrP^C (525). Indirect evidence suggested that LRP1 may bind PrP^C, although from the published data it is unclear whether direct binding of the two proteins actually takes place, or, alternatively, may be mediated by glycosaminoglycans or other intermediates. Nonetheless, cross-linking, coimmunoprecipitation, and direct binding experiments support the hypothesis that LRP1 does actually bind the NH_2-terminal domain of PrP^C (R. J. Morris, personal communication).

E. Copper and PrP^C

Copper constitutes a special case of confirmed PrP^C ligand, the physiological role of which is a matter of controversy. Early work showed that the NH_2-terminal $PrP^C_{59–91}$ domain, which contains four or five repeats of

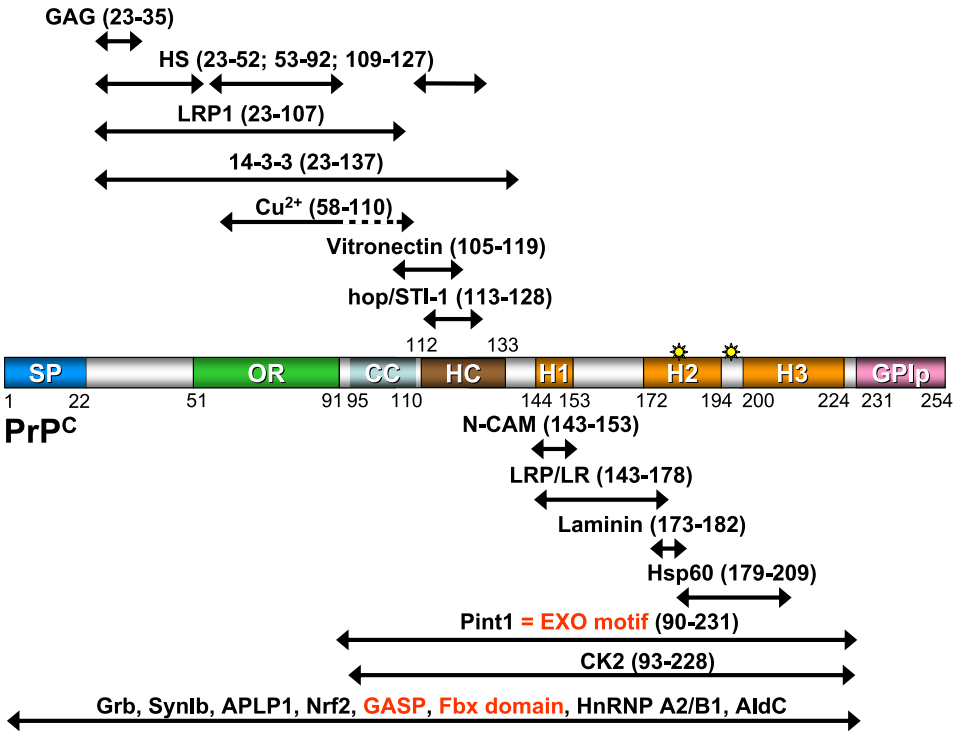

FIG. 3. PrP^C-binding partners (see text for details and references). The translated sequence of the prion protein is depicted as a rodlike shape, with major domains shown in color (amino acid residue numbers as for mouse PrP^C). SP, signal peptide; OR, octapeptide repeat domain; CC, charged cluster; HC, hydrophobic core; H1, H2, H3, α-helix domains; GPIp, GPI anchor-signaling peptide. Yellow stars indicate the position of the glycosylation residues, at amino acids 180 and 196. Each binding partner is indicated together with the stretch of amino acid residues that contain the binding domain in mouse PrP^C. GAG, glycosaminoglycans; HS, heparan sulfate; LRP1, low-densitiy lipoprotein receptor-related protein; LRP, laminin receptor precursor protein; LR, laminin receptor; Pint1, prion protein interactor 1; EXO, exonuclease domain; CK2, casein kinase 2; Grb, growth factor receptor-bound protein; SynIb, synapsin Ib; APLP1, amyloid precursor-like protein 1; Nrf2, nuclear factor E2-related factor-2; GASP, G protein-coupled receptor-associated sorting protein; Fbx, F-box only; HnRNP, heterogeneous nuclear ribonucleoprotein; AldC, aldolase C/zebrin. Data from either human, hamster, or bovine proteins were transposed to homologous mouse sequences.

eight aa residues (PHGGGWGQ), binds Cu^{2+} (52, 214, 215). Coordination of Cu^{2+} likely occurs with residues HGGGW, and imidazole coordination has been identified (12, 64, 361, 546); amide-nitrogen coordination from the Gly residues was also detected (64, 361), making the coordination site likely composed of three nitrogen and one oxygen atoms (546; see Ref. 359 for review).

In vitro, the octarepeats appear to strictly prefer Cu^{2+} over Cu^{+} and other metal ions (509, 546), but lower affinity binding of other metals has also been detected in full-length PrP^C (48, 236; see Refs. 94, 560 for reviews). Cooperative binding of four Cu^{2+} was shown in these octarepeats. However, some studies reported positive cooperativity (52, 65, 167, 576), whereas others have shown negative cooperativity with a Hill coefficient of 0.7 (552).

A range of binding affinities for interactions of PrP^C and Cu^{2+} were estimated, mostly within the low micromolar range (359). Binding of Cu^{2+} to PrP^C was reportedly the most effective at neutral pH, with reduced affinity at lower pH (363, 576, but see Ref. 567). This is consistent with a transport or sink function of PrP^C, because the protein could bind Cu^{2+} at the cell surface and release it inside acidic endosomal vesicles (283, 361, 414, 417, 576; see sect. III*D*). Nevertheless, direct evidence that PrP^C does in fact transport Cu^{2+} is still lacking.

On the other hand, other Cu^{2+} coordination sites were identified at His-96 (236) and His-111 (245) in human PrP^C, and it is possible that up to six Cu^{2+} can be present in a fully copper-loaded PrP^C (245). Moreover, recent experiments have indicated that, either at low Cu^{2+} occupancy or at low pH, there is a distinct coordination mode involving multiple histidine imidazole groups (567, 568). These can bind two Cu^{2+} with an estimated affinity in the nanomolar range, which has been proposed to be the biologically relevant mode of Cu^{2+} coordination, given the affinity of other proteins for copper (567).

Binding of Cu^{2+} changes PrP^C conformation (236, 362, 436, 437, 509, 546, 584), and folding of recombinant PrP^C is reportedly affected by Cu^{2+} (57, 245, 436, 584), although the physiological consequences of this change are not understood. Nonetheless, evidence has been reported that Cu^{2+} facilitates PrP^C self-association (567, 568).

Many reports showed a protective role of PrP^C against oxidative damage, which is perhaps the most widely ac-

cepted PrP^C function (52, 56, 202, 260, 438, 574, 583). Remarkably, loss of antioxidant defenses was suggested to play a major role in scrapie-infected cells (357) and prion diseases (9, 188, 189, 582).

Reactive oxygen species (ROS) increase lipid peroxidation and protein oxidation, leading to cellular damage. Free Cu^{2+} is highly cytotoxic, and must be minimized, because redox reactions of Cu^{2+} generate ROS (28). The antioxidant activity of PrP^C has been attributed to copper binding to the octarepeat region (47). For example, cultured PrP-null neurons were more vulnerable than wild type (WT) when exposed to high levels of Cu^{2+}, an effect that was blocked by treating cells with a synthetic $PrP^C_{59–91}$ peptide (55). Moreover, PrP^C can also be oxidized by Cu^{2+}, which could contribute to alterations in physiological functions and increased aggregation (381). Interestingly, anchoring the copper-binding NH_2 terminus of PrP^C to the plasma membrane using a signal peptide diminished oxidative responses in cells without affecting many aspects of PrP^C metabolism (595).

Copper content of brain membranes was reportedly lower in PrP-null mice, when compared with WT (46, 52, 202). Others, however, have failed to find decreased copper content in membranes from PrP-null mice (550), albeit some evidence exists that synaptosomal, rather than total brain, content of copper is decreased (46). Controlling the levels of PrP^C in an inducible cell system allowed regulation of Cu^{2+} binding to membranes, but not Cu^{2+} transport (438). Consistently, Cu^{2+} transport in synaptosomes appears to be independent of the expression of PrP^C (172).

It was also reported that cultured cells derived from PrP-null mice were more susceptible to oxidative damage and had reduced superoxide dismutase (SOD) activity, when compared with WT (56). Moreover, recombinant PrP^C refolded in the presence of Cu^{2+} was shown to have SOD activity (57). However, other authors found neither decreased SOD activity in PrP-null mice (550) nor SOD activity in recombinant PrP^C (462). Careful analysis comparing possible SOD activity in recombinant PrP^C with the activity of the SOD enzyme SOD-1 also failed to confirm any such activity for PrP^C (246). In addition, experiments using genetically modified mice, as well as crosses between PrP^C overexpressing mice and SOD-deficient and overexpressing mice, argue against such a role for PrP^C in vivo (227).

Alternatively, it is possible that the binding of Cu^{2+} induces endocytosis of PrP^C (see sect. III*D*) as a signal for triggering antioxidative defense (542). Reduced levels of glutathione reductase and other antioxidant enzymes have also been detected in PrP^C-deficient neurons (574). Conversely, increased PrP^C expression appears to augment such antioxidant defenses (438), and both oxidative stress and copper exposure appear to increase PrP^C expression (54, 541). These changes may constitute alternative mechanisms of PrP^C-dependent resistance to oxidative stress.

Therefore, although the binding of copper to PrP^C appears to impart cellular resistance to oxidative stress, the mechanisms are still controversial.

III. SUBCELLULAR ENVIRONMENT AND TRAFFICKING OF THE PRION PROTEIN

A. The Subcellular Environment of PrP^C

Notwithstanding minor truncated and transmembrane forms (see sect. III*B*), the GPI-anchored form constitutes almost all of PrP^C found during its normal life cycle (524). Similar to other GPI-anchored proteins, PrP^C molecules are found attached to low-density, detergent-insoluble membrane domains (DRM), rich in cholesterol and sphingolipids (382, 524).

DRMs are biochemically fractionated entities, usually equated with isolated lipid rafts and/or liquid ordered domains (370). The exact meaning of the terms *lipid raft* and *membrane raft*, as used over the years, is somewhat uncertain, and there is variable acceptance of distinct methods for preparing DRMs as representative of specific membrane domains (292, 370, 378). Membrane rafts have recently been consensually defined as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes and can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (422).

Despite these uncertainties, evidence points to a critical role of distinctive membrane domains upon the biology of the cell surface. Membrane rafts are involved in pathogen invasion, regulation of protein and lipid sorting, as well as in cellular signaling (45, 380, 415, 481, 537). The latter category highlights the dynamic nature of membrane signaling domains, which were shown to assemble from otherwise separate units upon engagement of surface proteins (273, 495).

Raft association appears to control the distribution of mature PrP^C among very distinct regions of the plasma membrane, as shown by the extensive redistribution of PrP^C at the surface of cultured neurons upon cholesterol or sphingolipid depletion (166). PrP^C coimmunoprecipitated with GM1 from neuroblastoma, and with GM3 from lymphoblastoid cells, both of which are markers of membrane rafts in their respective cell types (344). However, controversial findings were reported as both of the nature of PrP^C-containing rafts as well as of the distribution of PrP^C between raft and nonraft membrane domains.

In cultured dorsal root ganglia neurons, dissociated from 3- to 4-wk-old rats or Thy-1 transgenic mice, immunohistochemical labeling, detergent solubilization, and

immunoaffinity purification led to the conclusion that PrP^C-containing rafts are distinct from those containing Thy-1 (318). Further characterization of DRMs isolated from whole rat brain supported this hypothesis and showed that PrP^C DRMs contain significantly more unsaturated, longer chain lipids than Thy-1 DRMs, as well as other differences in lipid composition, consistent with the distinct solubility of the two GPI-anchored membrane proteins in nonionic detergents (61), as well as with the heterogeneity of membrane rafts (421).

Western blots of DRMs from rat cerebellar granule cells, followed by confocal microscopy of cultured neurons and coimmunoprecipitation, showed that, although both PrP^C and the acylated non-receptor tyrosine kinase Fyn are associated with DRMs, these two proteins in general do not colocalize in the membrane, whereas PrP^C associates with the growth-associated GAP43 protein (38).

In contrast to both studies above, no distinction between PrP^C, Thy-1, and Fyn-containing rafts was achieved in another study of DRMs isolated from rat cerebellar granule cells (304). It is unclear why studies employing a similar cell culture procedure achieved such distinct results.

In addition, association of PrP^C with lipid rafts is dynamic. A large fraction of the protein is found in nonraft membrane, on its way to coated pits (518; see sect. III*C*). Conversely, PrP^C can be recruited from a large pool of nonraft to raft membrane domains upon engagement of lymphoblastoid cells by cross-linked antibodies (225).

B. Trafficking of PrP^C to the Cell Surface

Experiments using brefeldin A showed that Golgi passage is needed for PrPs to reach the plasma membrane (520) and that exit of PrP^C from the Golgi and passage by the cell surface are required for the generation of de novo PrP^{Sc} (36, 82, 520). However, only recently has progress been made in the understanding of initial events of PrP^C synthesis, raft insertion and localization (Fig. 4).

PrP^C is translocated to the ER due to the presence of an NH_2-terminal signal peptide that is then cleaved into the ER lumen. Remarkably, PrP^C can be synthesized with at least three topologies in the endoplasmic reticulum (ER): a secreted form that reflects the main pathway for PrP^C synthesis in vivo, plus COOH- and NH_2-terminal transmembrane forms, ^{Ctm}PrP and ^{Ntm}PrP, respectively, due to transmembrane insertion of the hydrophobic pocket between aa 110–134 (197, 198). The major GPI-anchored form is derived from the secreted form, which fully translocates into the ER (197, 198). Interestingly, synthesis of the GPI-anchored protein depends on the presence of still unidentified *trans*-acting factors in the rough ER, whereas the ^{Ctm}PrP is favored when ER translocation is reconstituted in vitro with minimal components (197). The ^{Ctm}PrP was suggested to be toxic and cause degeneration, and certain mutations appear to favor this conformer (197), although ^{Ctm}PrP appears not to be infectious when inoculated in reporter mice (508).

Physiological roles of the transmembrane forms of PrP are not clear. However, transgenic mice expressing ^{Ctm}PrP develop neurological illness and neuronal death that resembles certain prion diseases (508). Since ^{Ctm}PrP accumulates in the Golgi apparatus in these mice, there is a possible role for Golgi dysfunction in the neuropathology of mice that express PrP^C mutants that favor ^{Ctm}PrP (507). It is noteworthy that the transmembrane domain in membrane inserted PrPs is similar to the region that binds certain PrP^C ligands, such as hop/STI1 (92, 591). This suggests that the membrane occlusion of this site may influence cell physiology, which would be consistent with the purported importance of PrP^C:hop/STI1 interaction (see sects. VII and VIII).

The significance of variable amounts of cytosolic PrP^C, detected usually after proteasome inhibition or overexpression of transfected *Prnp*, but also found in a subset of cerebellar neurons, is still debatable. It is not clear why WT PrP^C is occasionally retained in the cytosol, nor whether it serves any physiological function or, alternatively, may be associated with neuronal pathogenesis (72, 145, 312, 314, 360, 399, 555, 556). Indeed, in human neurons in culture, cytosolic PrP^C is not toxic and may, instead, have antiapoptotic functions (456). Recent work by Leblanc and collaborators (244) suggests that lack of translocation to cytosol for some PrP mutants affects their anti-Bax activity (244). Remarkably, cytosolic expression of PrP mutants that show defective anti-Bax activity restored the antiapoptotic function of the mutants, suggesting that these mutations may affect the ability of PrP^C to move to the cytosol, rather than affecting a direct antiapoptotic function of PrP^C (244). Although cytosolic PrP^C has been attributed to ER retrotranslocation (313), others have found that cytosolic PrP^C contains the NH_2-terminal signal peptide, indicating that this subcellular location may be exclusively related to abortive translocation into the ER (72, 134, 145).

The GPI anchor is added in the ER, after removal of a COOH-terminal peptide signal. The core GPI anchor added to the immature PrP^C is then processed (502), by a hitherto poorly understood mechanism.

Association of PrP^C with lipid rafts reportedly starts early within the ER, and immature (endoglycosidase H-sensitive) and mature (endo H-resistant) PrP^C are differentially sensitive to cholesterol or sphingolipid depletion, suggesting that maturation of PrP^C along the ER-Golgi-plasma membrane pathway is associated with a changing association with distinct membrane rafts (472). Re-

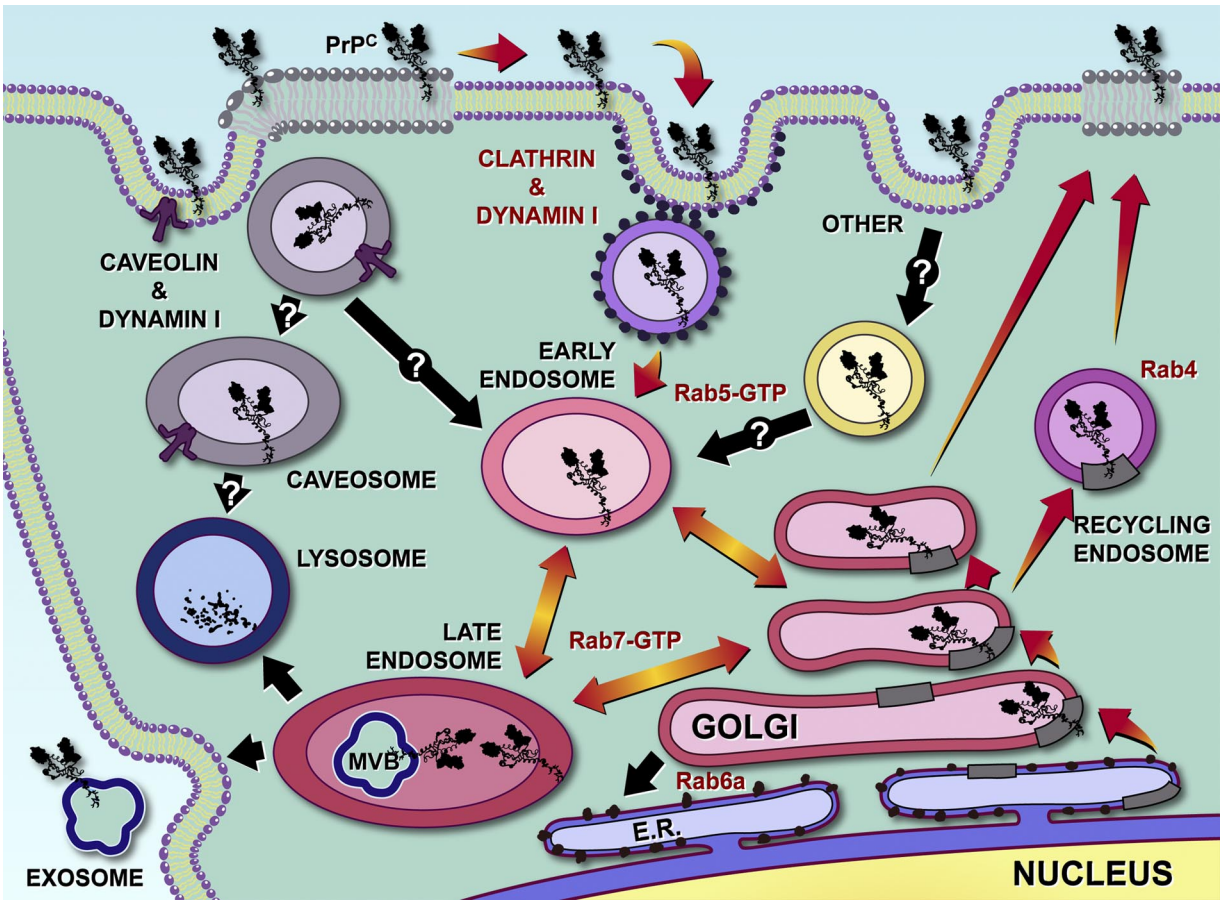

FIG. 4. Subcellular trafficking of PrP^C. The plasma membrane is represented by the lipid bilayer, intracellular membranes by continuous lines, and lipid rafts by a grey-shaded thicker bilayer. The cytoplasm is colored light green. PrP^C is depicted as a forklike representation of amino acids 124–231 (235), where the polypetide backbone and the GPI anchor are drawn in grey and the carbohydrate residues are shown as bunches of black spheres at glycosylation sites. The major vesicular systems involved in both the synthesis (*bottom right*), the endocytic cycle of PrP^C, and release of PrP^C-containing exosomes (*bottom left*) are indicated with black lettering. MVB, multivesicular bodies. Molecules involved in the clathrin-dependent pathway are indicated in red lettering. Arrows indicate trafficking, the most likely pathways based on current experimental evidence are shown with red arrows. Clathrin is represented by dark blue circles, caveolin is depicted in purple. Note that PrP^C is associated with rafts early in the secretory pathway and that cell surface PrP^C leaves the rafts to be internalized via clathrin.

sults of cholesterol depletion also suggested that raft association is required for correct folding of PrP^C (472), as well as for the export of the protein to the Golgi and proper glycosylation (73, 472). Moreover, PrP^C that does not associate with rafts in the ER appears to undergo conformational changes that modify protease sensitivity, indicating that the immature protein may be misfolded and subject to the ER quality control mechanism (472).

The glycosylation patterns of PrP^C may also affect protein trafficking and biophysical features. This was tested using transgenic mice expressing PrP^C mutants that were monoglycosylated either on Asn-180 or Asn-196, or nonglycosylated (74). Neurons from mice that produce only nonglycosylated PrP^C presented a shift in the localization of the protein, with accumulation in the Golgi (74, 581). Interestingly, no overt phenotype, neurological signs, or neurodegeneration were found in these mice, indicating that simple accumulation of PrP^C in the Golgi may not necessarily be deleterious.

C. Endocytosis and Internalization of PrP^C

Initial interest on trafficking of PrP^C was raised by the finding that PrP^{Sc} (or PrP^{res}) was derived from a GPI-anchored cellular precursor, which journeys to the cell surface and to endosomal vesicles prior to conversion (38, 82). Indeed, evidence for endosomal conversion of PrP^C into protease-resistant forms has received considerable attention in recent years (see, for example, Ref. 80).

Many GPI-anchored proteins are sequestered into intracellular compartments (346, 347, 384, 412). Earlier work showed that chicken PrP^C transfected into a neuroblastoma cell line cycled between the plasma membrane

and endosomal compartments with a $t_{1/2}$ of 20–30 min (486). In neurons, endogenous PrP^C appears to internalize as fast as classical membrane receptors, such as the transferrin receptor, with a $t_{1/2}$ of ~3–5 min (518).

A controversy regarding the role of clathrin- and non-clathrin-coated vesicles in the internalization of PrP^C (424) was fueled by the idea that GPI-anchored proteins use a lipid raft-based mechanism for internalization (250), because they lack transmembrane domains able to interact with clathrin adaptor proteins. Moreover, localization of both PrP^C and PrP^{Sc} in rafts, or caveolae-like domains, was also reported and led to the contention that clathrin-coated vesicles did not participate in PrP^C trafficking (250, 544). Evidence for PrP^C internalization via non-clathrin-coated vesicles was, indeed, obtained in nonneural cells and also in glia (335, 419).

A recent electron microscopic study of PrP^C internalization in CHO cells used protein A-gold to follow PrP^C internalization into caveolae, since the authors indicated that protein A-gold, but not protein A-FITC, binds PrP^C in transfected cells (419). However, this unconventional way to follow PrP^C trafficking, added to the use of nonneural cells, precludes generalization. Since GPI-anchored proteins may follow distinct endocytic routes depending on both the cell type and membrane environment (147), the results obtained in CHO cells likely do not apply to other specialized cell types, such as neurons. In fact, despite its GPI-dependent raft association, sequestered PrP^C was soluble in nonionic detergents, a common criterion for residence outside raft membranes. Therefore, rather than internalization via rafts, PrP^C appears to move to nonraft membrane to be internalized (518).

Although PrP^C lacks the expected intracellular internalization signals, mounting evidence supports a major role of clathrin-coated vesicles in the internalization of PrP^C (Fig. 4). The pioneer evidence that chicken PrP^C is internalized via clathrin-coated vesicles (485) has now been confirmed and extended to mammalian PrP^C (518, 525, 526). Cell surface biotinylation, live cell microscopy, GFP-tagged PrPs, and electron microscopy support the view that clathrin-coated vesicles and classical endosomal organelles are involved in endocytosis of PrP^C (59, 321, 485, 518, 526). Dominant negative approaches indicated a role for the activities of dynamin (321) and clathrin in the internalization of PrP^C in distinct cell lines (526).

A mechanism was identified by which raft-associated PrP^C can be sequestered by clathrin-coated vesicles. It was proposed that an NH_2-terminal, positively charged domain of PrP^C (KKRPKP) is responsible, perhaps by interacting with a transmembrane protein, for the constitutive endocytosis of PrP^C by clathrin-coated vesicles (518). Remarkably, a number of reports indicated a role for the NH_2-terminal region of PrP^C upon endocytosis and cellular trafficking (283, 392, 488), and this basic region of the protein has been previously implicated in the binding of negatively charged proteoglycans, which are thought to modulate PrP^C sequestration (80).

The hypothesis that GPI-anchored PrP^C may "piggyback" on an integral membrane protein had long been raised (485), similar to the mechanism of interaction between the GPI-anchored urokinase receptor and a low-density lipoprotein receptor-related protein (LRP1),[1] a transmembrane protein that can interact with adaptor proteins (112, 119, 391). Indeed, recent experiments indicated that LRP1 may participate in clathrin-mediated PrP^C endocytosis, because knock-down of LRP1, but not LRP1b, reduced internalization of PrP^C (525; see also Ref. 371).

In addition to these findings, blockade of PrP^C internalization by antibodies to the LRP in various cells, as well as transfection of deleted mutants, suggests that LRP and/or the laminin receptor may play a major role in the subcellular traffic of PrP^C (170, 171). Nonetheless, the latter experiments were done with a recombinant soluble PrP^C, rather than the GPI-anchored protein, and LRP was able to mediate internalization of only part of the membrane-bound recombinant protein (171), which is consistent with the participation of other cell surface proteins in the trafficking of PrP^C.

High extracellular levels of Cu^{2+} induce the endocytosis of PrP^C to intracellular organelles and the Golgi (59, 283, 414, 417). It was reported that Cu^{2+}-induced endocytosis of PrP^C expressed in neuroblastoma cells caused its movement from raft to nonraft membrane regions. The KKRP motif was shown to be important for endocytosis, supporting earlier findings for the constitutive endocytosis of endogenous PrP^C (526). Interestingly, this KKRP motif is not essential for the lateral displacement of PrP^C to nonraft membrane, indicating that this movement occurs prior to PrP^C endocytosis (526). It was also suggested that Cu^{2+} may destabilize a putative PrP^C interaction within rafts, rather than inducing PrP^C to interact with a nonraft protein (526).

Further analysis along these lines should allow the understanding of the fundamental mechanisms through which the GPI-anchored PrP^C may be internalized by clathrin-coated vesicles.

D. Modulation of the PrP^C Endocytic Cycle

In addition to copper (see above), both sulfated glycans and suramin, compounds that affect scrapie replication, may also induce internalization of PrP^C (173, 258, 487).

It was proposed that changes in PrP^C conformation induced by suramin and Cu^{2+} may lead to internalization

[1] Not to be confounded with the laminin receptor precursor, LRP.

(258). Whether copper-induced endocytosis of PrP^C is an acceleration of a physiological mechanism is unclear, although many of its features are similar to those of the constitutive internalization of PrP^C, such as the involvement of clathrin and the need for exit from lipid rafts (518, 526).

If exit from lipid rafts, rather than misfolding, is the trigger for PrP^C internalization, engagement of PrP^C with ligands that affect its lateral distribution may also induce endocytosis. PrP^C appears to reside in the most external shell of the rafts (318, 371), the protein remains only for a relatively short period of time at the cell surface (486; reviewed in Ref. 424), and indeed, PrP^c cross-linking in lymphoid cells induced endocytosis (512). However, it is still unknown whether the endocytic cycle of PrP^C may depend on a physiological ligand.

It is likely that cytosolic factors that participate in clathrin-mediated endocytosis, such as the small GTP binding Rab proteins (596), may have a key role in controlling PrP^C trafficking (Fig. 4). Rab 5 is a candidate (321), but Rab 7-positive organelles (late endosomes and lysosomes), which participate in PrP^{Sc} invasion of cells, may also play a role (320). Other Rab proteins, such as Rab 6, involved with retrograde transport of PrP^{Sc} to the ER, may also regulate trafficking of PrP^C (26).

Proteolytic cleavage of PrP^C occurs during its cycle between intracellular compartments and the cell surface, and clipping usually removes the NH_2-terminal region, involved in endocytosis (see above), and possibly also in raft localization (551). Thus the processed COOH-terminal domain of the protein accumulates at the cell surface (488) and may affect sensitivity to cell death (517).

Major unanswered questions remain as to the physiological regulation of the endocytic cycle of PrP^C. This subject merits further attention, especially because of its possible involvement in physiological PrP^C-mediated signaling mechanisms (see below). An important issue refers to the fate of recycled PrP^C, following each round of internalization. A small fraction of endocytosed PrP^C is degraded by lysosomes, but large fractions return to the cell surface. In addition, work originally intended to understand the intercellular transfer of PrP^{Sc} has led to evidence that part of the recycled PrP^C may be secreted to the extracellular medium associated with exosomes (144, 423, 447). These membrane-bound vesicular structures are derived from multivesicular bodies formed within the endosomal system and have been associated with transfer of molecules, for example, between immune cells (see Ref. 423 for review). This may allow bidirectional cellular interactions, mediated either by the binding of extracellular ligands (sect. II*D*) to cell-surface PrP^C, as well as the binding of secreted PrP^C to cell-surface ligands (see sect. IX).

IV. FUNCTIONS OF THE PRION PROTEIN IN THE NERVOUS SYSTEM

A. The First Knockouts and the Lack of Phenotype

Molecular cloning of the cDNA coding the entire ORF of mouse *Prnp* (305) and the use of homologous recombination allowed the generation of mice in which the *Prnp* gene was deleted. All PrP-null animals generated to date established a tight correlation between the presence of PrP^C and the sensitivity to prion infection, and support the model of propagation of PrP^{Sc} by co-option of PrP^C. This has been largely discussed in the literature (566 for review) and is outside the scope of this article.

The first PrP-null mouse strain was produced in a mixed C57BL/6J × 129/Sv(ev) background, by replacement of codons 4–187 with a neomycin phosphotransferase (*neo*) expression cassette (63). These animals, designated $Prnp^{0/0}$ or Zurich I (ZrchI), had no gross anatomical abnormalities in the brain, nor in skeletal muscle and visceral organs. Morris' water maze, Y maze discrimination, and a test that measures the animal's ability to compare the efficiency of distinct problem-solving strategies showed no defects in behavior or learning (63). Unfortunately, the mixed background has been a barrier for the interpretation of several more recent experiments, particularly those related to behavior.

A second line of PrP-null mice was produced by interruption of the *Prnp* ORF at position 93 (a Kpn 1 site) and introduction of a *neo* cassette (331). These mice are known as Npu, $Prnp^{-/-}$ Edinburgh (Edbg), and are under 129/Ola genetic background. In agreement with the previously generated ZrchI mice, the Edbg mice did not present any developmental alterations.

Taken together, the first results obtained with two distinct PrP-null mouse strains suggested that either PrP^C is unnecessary for normal development, or its absence is compensated by a redundant protein which maintains an important phenotype (63, 331).

B. Neurodegeneration in PrP-Null Mice

In sharp contrast to the preceding strains, PrP-null mice were generated that developed normally but underwent severe ataxia and Purkinje cell degeneration at advanced ages. This was first observed in *Prnp*-ablated mice now known as Nagasaki (Ngsk), in which besides the *Prnp* ORF, 0.9 kb of intron 2, 10 bp of the 5′-noncoding region, and 0.45 kb of the 3′-noncoding region were also replaced by a *neo* cassette (461). Since the observed

phenotype was abolished by reintroduction of *Prnp*, it was attributed to the absence of PrP^C (389).

The same *Prnp* deletion approach and replacement by another resistance marker, the hypoxanthine phosphoribosyl transferase gene, led to the same degenerative phenotype in mice now known as Rcm0 (366). Notably, similar results were obtained when, in addition to the *Prnp* ORF, 0.26 kb of intron 2, 10 bp of the 5′-noncoding region, the 3′-noncoding region of exon 3 plus 0.6 kb of the adjacent region, were deleted in Zurich II (ZrchII) mice (453).

C. The Knockout Paradox, Doppel Gene, and Neurodegeneration

The constructions used to generate animals that suffered cerebellar degeneration and ataxia differ from those that lacked phenotypic effects (63). In the former, large portions of the ORF flanking regions were removed. The remarkably distinct phenotypes of Ngsk, Rcm0, and ZrchII mice, when compared with ZrchI and Npu, were obviously associated with the deletion approach (563).

In fact, it was demonstrated that ataxia in these animals was caused by overexpression of a protein that received the name Doppel, Dpl, coded by the gene *Prnd*, located 16 kb downstream of *Prnp* (366). The *Prnd* promoter is weak in the brain, but when the *Prnp* deletion extends to exon 3, its splice acceptor site is lost, leading to exon skipping and the generation of a chimeric mRNA containing the first two noncoding *Prnp* exons plus the Dpl-encoding *Prnd* exon. Thus *Dpl* becomes controlled by the *Prnp* promoter and therefore highly expressed in brain (288, 366).

Dpl is overexpressed in Ngsk, Rcm0, and Zrch II mice, but not in Zrch I or Npu animals (366). The ectopic expression of Dpl in Zrch I mice caused ataxia and degeneration of cerebellar granule and Purkinje cells, and its levels were inversely correlated with the onset of disease (367). Indeed, these results showed that ectopic expression of Dpl, rather than the absence of PrP^C, caused neurodegeneration. Notably, the reintroduction of *Prnp* gene in mice overexpressing Dpl in the brain rescued the phenotype (367, 389). Thus Dpl neurotoxicity is counteracted by PrP^C, but the mechanisms underlying this antagonism remain elusive (reviewed in Ref. 23).

D. Neurodegeneration in Other Transgenic Mice

Neurodegeneration is not uniquely associated with overexpression of Dpl (Fig. 5). Transgenic mice overexpressing PrP^C were generated from various constructions. The first was a large cosmid containing the three exons and two introns of the mouse $Prnp^b$ allele (108Phe, 189Val) and encompasses 6 kb of the upstream sequence plus ~18 kb of the 3′-downstream sequence (572). A second construction, named *half-genomic* DNA, was derived from the previous cosmid by deleting intron 2 and replacing the exon 3 by the $Prnp^a$ allele (108Leu, 189Thr) and the 18 kb of the 3'-sequence by a 2.2 kb downstream sequence from the $Prnp^a$ locus (146). Mice overexpressing WT PrP^C coded by the first construction developed ataxia, hindlimb paralysis, and tremors, while those expressing high levels of PrP^C from the half-genomic construction had no obvious phenotype (146, 527, 570).

The large cosmid construction cited above contains the *Prnd* exon codifying Dpl protein. However, toxic effects mediated by Dpl can be ruled out in this model, since mice expressing the hamster PrP transgene from a similar cosmid, in which the *Prnd* exon is lacking, also developed ataxia (570). In addition, the results were not due to positional effects of integration of the transgene, since the phenotype was found in several independent strains of *Prnp* cosmid transgenic mouse.

The distinct phenotypes of these mice may be due to the allelic nature of the two transgenes. It is known that alleles $Prnp^a$ and $Prnp^b$ have been associated with either long or short disease incubation times, respectively (571). Mouse PrP^C coded by alleles $Prnp^a$ and $Prnp^b$ contain Leu or Phe at codon 108, respectively. Both amino acids are nonpolar and neutral, but Phe holds a longer lateral chain than Leu. On the other hand, $Prnp^a$ allele codes for polar Thr at position 189, whereas Val in allele $Prnp^b$ is nonpolar. The chemical properties of these amino acids could possibly affect either the interaction between PrP^C and its cellular ligands (see sect. II*D*) or recognition of PrP^C by proteases, leading to changes in cellular processes such as sensitivity to cell death (517). These possibilities deserve further investigation in the context of PrP^C physiology.

The half-genomic PrP vector was also used to clone deletion mutants of the $Prnp^a$ allele, which were then expressed as transgenes in Zrch I $Prnp^{0/0}$ animals (Fig. 5). Interestingly, although the reexpression of either full-length PrP^C or mutants with deleted aa 32–80, 32–93, or 32–106 in $Prnp^{0/0}$ mice did not cause an altered phenotype, deletion of aa 32–121, or 32–134, led to both severe ataxia and apoptosis in the cerebellum of relatively young animals (484). This latter phenotype was rescued by introducing one copy of a WT *Prnp* gene (484).

The phenotype observed in mice expressing $PrP_{\Delta 32–134}$ resembles animals that overexpress Dpl. Since the globular domains of PrP^C and Dpl are similar, and both Dpl and the PrP^C deletion mutants lack the flexible NH_2-terminal domain, it was suggested that the mechanisms of neurodegeneration by both Dpl and $PrP_{\Delta 32–134}$ might be the same (565). Remarkably, however, while Dpl leads to massive degeneration of cerebellar Purkinje cells (461), $PrP_{\Delta 32–134}$ caused degeneration in the granule cell layer (484).

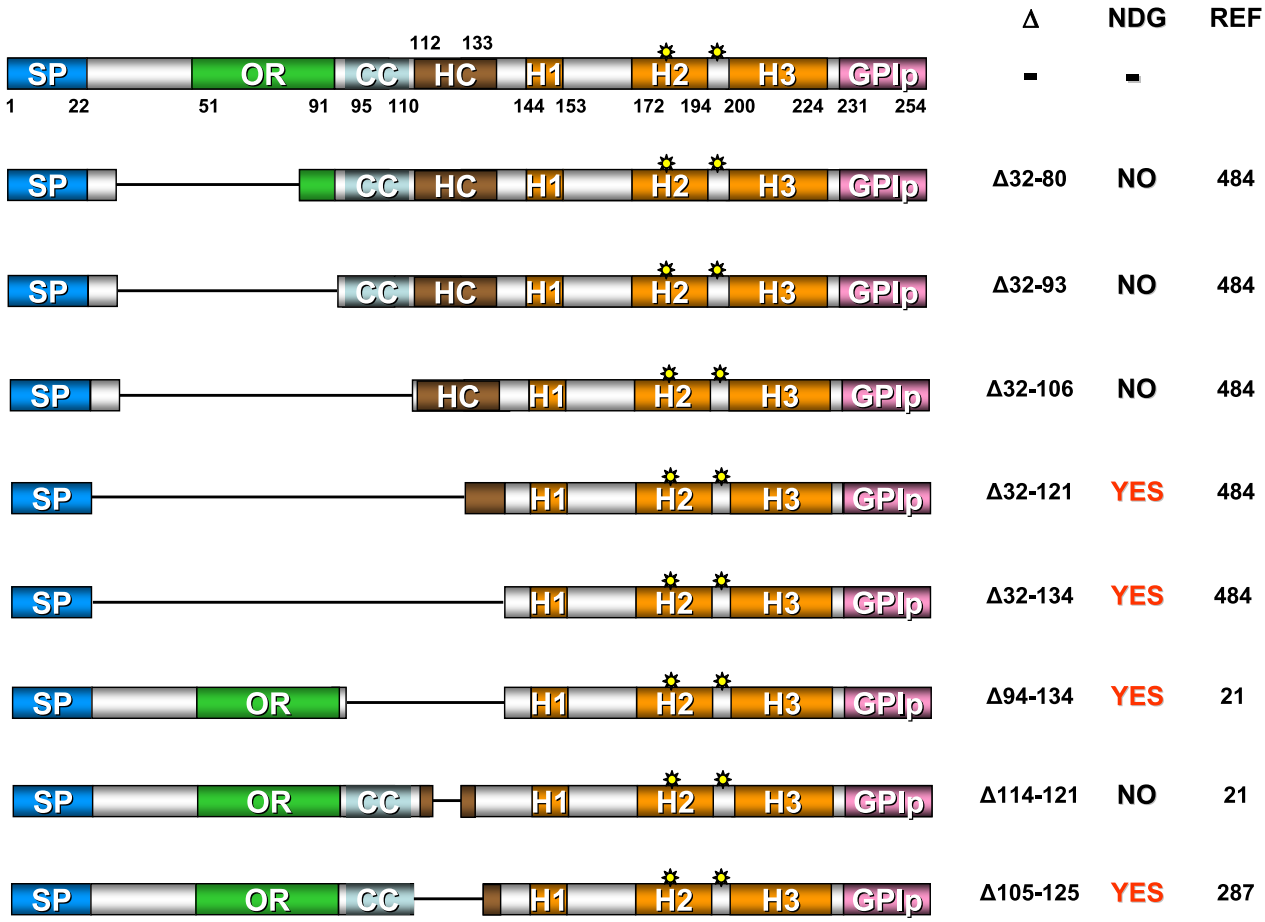

FIG. 5. PrP^C deletion mutants expressed in $Prnp^{0/0}$ mice and their neurodegenerative phenotype. The wild-type PrP^C molecule with major domains annotated is shown at the *top*, as in Fig. 3. Deleted domains are replaced by a solid black line, and the deleted amino acids are indicated to the *right* of each diagram (Δ). The presence or absence of neurodegeneration (NDG) is also indicated, as well as the respective reference (REF).

The half-genomic construction partially mimics the endogenous expression pattern of PrP^C, but it promotes high expression of PrP^C in granule cells, while expression in Purkinje cells is undetectable (93, 146). On the other hand, Dpl under control of the *Prnp* promoter is highly expressed in Purkinje cells, in contrast to lower expression in granule cells (288, 453). Thus specificity of neuronal death induced either by truncated PrP^C in granule cells, or by Dpl in Purkinje cells, may be associated with their differing levels of expression in distinct neurons. This hypothesis is supported by the elegant demonstration that truncated $PrP_{\Delta 32–134}$ specifically targeted to Purkinje cells of ZrchI $Prnp^{0/0}$ mice led to their selective degeneration, whereas the granule layer was unaffected (148).

Mice expressing either Dpl, or one of the truncated forms $PrP_{\Delta 32–121}$ or $PrP_{\Delta 32–134}$, presented severe demyelination and axon loss in the spinal cord and cerebellar white matter (439). The leukodystrophy, but not the cerebellar granule cell degeneration (484), was rescued by oligodendrocyte-specific expression of PrP^C. On the other hand, neuron-specific expression of PrP^C partially rescued cerebellar granule cell degeneration but not demyelination. Thus white matter disease and cerebellar granule cell degeneration in these mice are distinct, and endogenous expression of PrP^C in both neurons and glia was required for complete reversion of the degenerative phenotype (439).

In recent studies, transgenic mice expressing truncated $PrP_{\Delta 105–125}$ (Fig. 5) developed an extremely severe illness within 2 wk of birth, with decreased body size and weight, immobility, impaired righting, myoclonus, and tremor and died within 1 mo. Histopathology demonstrated cerebellar atrophy, severe loss of cerebellar granule cells, gliosis, and astrocytic hypertrophy. Remarkably, older symptomatic mice expressing the truncated protein associated with the Tga20 allele (which overexpresses PrP^C) in hemizygosis, presented vacuolar degeneration of the white matter of both the spinal cord and the brain, but did not undergo cerebellar degeneration (287).

Simultaneously, mice expressing a truncated $PrP_{\Delta 94–134}$ form (Fig. 5) showed extensive central and peripheral myelin degeneration and developed early ataxia, which progressed to spastic paraparesis and full paraplegia (21).

Interestingly, this lethal phenotype was not associated with the presence of pathological aggregates, altered PrP^C glycosylation, subcellular mislocalization, or inappropriate membrane topology. The phenotype was rescued dose dependently either by WT PrP^C or a PrP^C lacking all octarepeats. Mice expressing a smaller truncation $PrP_{\Delta 114-121}$ showed no phenotype either in the presence or absence of WT PrP^C. Interestingly, however, the expression of $PrP_{\Delta 114-121}$ ameliorated the phenotype caused by truncated $PrP_{\Delta 32-134}$, but enhanced the degenerative effect of $PrP_{\Delta 94-134}$ (21).

The mechanisms of neurodegeneration mediated by the expression of Dpl, $PrP_{\Delta 32-134}$, $PrP_{\Delta 94-134}$, and $PrP_{\Delta 105-125}$ are still elusive. An early hypothesis (484, 565) was that PrP^C binds to a putative ligand, inducing specific cell signaling that mediates neuronal survival. In the absence of PrP^C, a redundant homolog molecule, with lower affinity for the putative PrP^C ligand, would replace PrP^C, therefore explaining the lack of neurodegeneration in PrP-null mice. On the other hand, NH_2-terminal truncated PrP^C or Dpl molecules might compete with this PrP homolog for the putative ligand without eliciting survival signals (484, 565). Alternatively, truncated PrP and Doppel may activate pathways of programmed cell death that are normally kept quiescent either by PrP^C or by its functional homolog (148). The recent studies of centrally truncated PrP transgenics (21, 285) led to distinct hypotheses, based on the putative interaction of truncated PrP^C with a single PrP^C receptor.

One proposal suggests that myelin integrity depends on dimerization of PrP^C, leading to dimerization of the putative PrP^C receptor. Dimers of truncated $PrP_{\Delta 32-134}$ would trap the putative receptor in a dominant-negative form, which fails to induce signals for myelin maintenance. The presence of the octarepeat domain in the truncated $PrP_{\Delta 94-134}$ is thought to generate a more stable dominant-negative form of the receptor, causing more severe pathology than truncated $PrP_{\Delta 32-134}$ (21).

Another hypothesis is that the more severe neurodegeneration in mice expressing the truncated $PrP_{\Delta 105-125}$, when compared with $PrP_{\Delta 32-134}$ (285), is associated with a higher affinity of the former for a putative receptor. In normal conditions this receptor would transduce neuroprotective signals, but its altered conformation when coupled with the centrally truncated PrP^C, would lead to neurotoxic signals. The authors also proposed that the so-called neurotoxic peptide $PrP^C_{105-125}$ may compete with PrP^C for this receptor, thereby inducing toxicity (285). However, this seems unlikely in view of the apparent requirement that the neurotoxic peptide must be aggregated to produce toxicity (92, 140).

Interestingly, while $PrP_{\Delta 105-125}$ in PrP^C-null background produced degeneration particularly in the cerebellum (285), $PrP_{\Delta 114-121}$ in the same background was innocuous (21). Thus PrP^C domains from aa 105–114 and 121–125 may exert important modulatory activity. Moreover, the most prominent phenotype observed in animals expressing truncated $PrP_{\Delta 105-125}$ was cerebellar cell death, whereas $PrP_{\Delta 93-134}$ caused massive myelin degeneration, indicating that the PrP^C domains including amino acids 93–104 and 126–134 may have an impact on specific mechanisms underlying myelin integrity.

The idea of a PrP^C homolog/redundant molecule which mediates neuronal survival in the CNS (484) is plausible, but its identity is unknown. Notably, in the peripheral nervous system, PrP^C mediates neuronal differentiation through its interaction with vitronectin, which is compensated by integrins in PrP-null cells (192). Nevertheless, there is as yet no evidence for a role of integrins in compensatory mechanisms associated with either neuronal survival or myelin integrity in PrP-null neurons.

As for the elusive PrP^C receptor alluded to in the recent studies (21, 285), among the various PrP^C ligands identified to date (see sect. II*D*), at least hop/STI1, the interaction of which with PrP^C mediates neuroprotection (92, 591, see sect. VII*C*), binds to the domain $PrP^C_{113-128}$, which is deleted in the truncated molecules with neurodegenerative activity. However, there is no evidence that hop/STI1 is the putative PrP^C ligand/receptor proposed in the different models (21, 285, 484). In addition, the vitronectin binding site in PrP^C maps between aa 105–119, which are also within the domain the deletion of which promotes neurodegeneration. Nonetheless, the role of PrP^C-vitronectin interaction in neuronal survival was not tested (192). It is, in fact, unlikely that a single PrP^C-binding molecule may explain the degenerative phenotypes induced by all the truncated forms described to date. A conservative hypothesis is that various truncated PrP^C forms may lead to distinctive patterns of cell surface protein complexes formed by its ligands, which differ from the protein complexes, that a full-length, normal PrP^c molecule may be able to sustain (see sect. IX).

E. Role of PrP^C in Behavior

One of the first phenotypes attributed to the lack of PrP^C was the impaired motor coordination of aged Ngsk PrP-null mice, which correlated with the loss of cerebellar Purkinje cells (253). However, these findings are associated with increased expression of Dpl in the brain (see above), rather than loss of function of PrP^C.

On the other hand, ZrchI *Prnp*$^{0/0}$ mice showed a slight increase in locomotor activity during exploration of a novel environment (451). These mice also showed reduced hyperlocomotor responses to the NMDA receptor antagonist MK-801, but normal responses to either amphetamine or caffeine, when compared with WT. This suggested that the lack of PrP^C leads to functional changes in the

glutamatergic system, whereas both dopaminergic and adenosinergic systems are preserved (102).

Under acute stress, such as restraint or electric foot shock, ZrchI *Prnp*$^{0/0}$ mice showed reduced levels of anxiety, when compared with WT controls (386). Anxiety is accompanied by a characteristic set of behavioral and physiological responses that tend to protect the individual from danger and is taken as part of a universal mechanism of adaptation to adverse conditions (reviewed in Ref. 187). The lower levels of anxiety in *Prnp*$^{0/0}$ animals suggest that PrP^{C} may help the adaptation to stress at the systems level (386).

F. PrP^{C} and the Sleep-Wakefulness Cycle

The circadian clock relies on molecular clocks and their key regulated genes. Mutations or polymorphisms in human clock genes are linked with several sleep-phase syndromes (194). Several studies are consistent with a role of PrP^{C} in the control of the sleep-wakefulness cycle.

Fatal familial insomnia (FFI) is clinically characterized by progressive insomnia, dysautonomia, and motor signs and was associated with severe atrophy of the anterior ventral and mediodorsal thalamic nuclei (309). The brains of most members of an affected family showed gliosis of the cerebral cortex, a moderate degree of cerebellar atrophy, and severe atrophy of the inferior olivary nuclei. One case also showed spongiform degeneration of the cerebral cortex. These findings raised the possibility that FFI belongs to the group of prion diseases (329). In fact, protease-resistant PrP was found in FFI patients (353), and the disease was successfully transmitted to experimental animals, confirming that FFI is an infectious cerebral amyloidosis (523). In addition, all affected members of the kindred had a point mutation in *PRNP* (the human PrP^{C} gene) codon 178 that substitutes asparagine for aspartic acid. Linkage analysis showed a close relationship between the point mutation and the disease (353), which was confirmed in a second kindred (352).

Nonetheless, substitution of Asn for Asp at codon 178 also segregates with CJD (177, 178). Interestingly, both FFI and familial CJD segregate with a combination of the Asn-178 mutation and the Met-Val polymorphism at codon 129. The Met-129/Asn-178 allele segregated with FFI, whereas the Val-129/Asn-178 allele segregated with familial CJD. Thus a common polymorphism determines disease phenotypes linked to the same pathogenic mutation (179).

Altered circadian rhythms and sleep patterns were found in PrP-null mice, raising the hypothesis that FFI resulted from PrP^{C} loss of function. Both ZrchI *Prnp*$^{0/0}$ and WT (C57Bl/6 $\times$ 129Sv) presented similar motor activity under light-dark conditions (12/12 h). However, a longer and more stable period of circadian motor activity was found in *Prnp*$^{0/0}$ mice under constant darkness, when compared with WT (535). Overexpression of PrP^{C} in transgenic Tga20 mice (146) restored the WT phenotype (535). Similar results were obtained with Npu mice (Edbg *Prnp*$^{-/-}$), when compared with their 129/Ola controls (535), strongly indicating that the changes in PrP-null mice are due to PrP^{C} depletion, instead of genetic background. Interestingly, PrP^{C} mRNA is regulated in a marked circadian manner, in both the suprachiasmatic nuclei and other forebrain regions (69).

Sleep is regulated as a function of prior wakefulness (34, 35), and electroencephalogram (EEG) slow-wave activity (SWA) is used as an index of sleep intensity (34). PrP-null mice from both ZrchI and Npu strains presented a lower amount of SWA during the episodes of non-rapid-eye-movement (NREM) sleep, whereas rapid-eye-movement (REM) sleep was unaffected (222, 534).

Mice show an increase of SWA after sleep deprivation (reviewed in Ref. 533). Notably, the increase of SWA during recovery sleep was twice as long in both ZrchI and Npu mice than in their respective WT (222, 534). Although these results are difficult to interpret, they suggested that PrP-null animals may have a low sleep pressure, leading to more frequent interruptions of sleep and reduced SWA (222, 534, 535). Tga20 PrP^{C}-overexpressing mice presented sleep fragmentation and a response to sleep deprivation similar to WT, indicating that reexpression of PrP^{C} rescued the altered phenotype of PrP-null mice (534, 535).

The EEG of mice shows regional differences during recovery from sleep deprivation (223). In a further experiment, Npu *Prnp*$^{-/-}$ mice presented a larger increase of SWA than WT in the occipital, but not in frontal derivation. The difference between both genotypes appeared after the waking-NREM sleep transition, indicating that PrP^{C} is not required for the transition to sleep (224). It was suggested that the differing sleep EEG between WT and PrP-null mice reflects either a direct influence of PrP^{C} on neurotransmission, or its protective role upon synapses (224). It is also possible that PrP-null mice are more susceptible to stress related to sleep deprivation, or even that the absence of PrP^{C} in the neuronal membranes may affect the generation of slow waves (222).

Besides FFI, intractable insomnia was reported in patients with a mutation at codon 200 responsible for a familial form of CJD (85, 521), in up to 45% of the patients with confirmed sporadic CJD (255, 529, 547), and in iatrogenic CJD (68). Sleep alterations may have been underestimated in prion diseases, and these signs may reflect a similar underlying mechanism in both CJD and FFI, associated with prominent histopathological changes in cortical areas, but independent on selective neuronal loss in thalamic nuclei (276).

G. PrP^C and Memory

The first experiments in PrP-null ZrchI mice failed to demonstrate altered memory (63). However, a role of PrP^C in memory retention was postulated, based on studies of PrP-null Ngsk mice (388). These animals presented impairment in latent learning at 24 wk of age, with normal learning ability and short-term memory (STM) retention, but a significant deficit in long-term memory (LTM) consolidation. Again, however, the high levels of Dpl (366) preclude an association of this deficit with PrP^C loss of function.

However, although Zrch I mice presented both normal STM and LTM at the age of 3–4 mo (105, 451), memory impairment was observed at 9 mo (105). Npu PrP^C null mice in either pure 129/Ola background or a mixed 129/Ola × C57Bl/10 background also exhibited impaired hippocampal-dependent spatial learning, while nonspatial learning remained intact. These deficits were rescued when PrP^C was selectively reexpressed in neurons, indicating that they were caused by loss of function of neuronal PrP^C (116).

Studies of genetically modified animals are controversial, largely because the gene deletion approach, genetic background and compensatory mechanisms, may all influence cognitive performance. Thus the use of pharmacological approaches offers distinct advantages for evaluating the roles of selected molecules upon cognitive processes, especially in memory consolidation (234).

Intrahippocampal infusion of an antagonist antibody to PrP^C impaired both STM and LTM retention in old rats, when compared with age-matched animals infused with an irrelevant IgG (105). Moreover, infusion of antibodies against either PrP^C or laminin (LN), or of peptides representing their respective binding sites (see sect. II*D*), impaired memory consolidation.

In rats trained in one-trial inhibitory avoidance, memory retention was impaired by an immediate, posttraining intra-CA1 infusion of either $PrP^C_{173–192}$, a peptide that contains the LN binding site or of anti-PrP^C antibodies that inhibit PrP^C-LN interaction. The amnesic effects of both anti-PrP^C antibodies and $PrP^C_{173–192}$ were reversed by coinfusion of the LN γ-1 peptide, that is the PrP^C-binding site in LN (see sect. II*D*). In addition, both anti-LN and anti-PrP^C antibodies also inhibited the activation of hippocampal PKA and ERK1/2, two kinases that mediate the upregulation of signaling pathways needed for the effective consolidation of inhibitory avoidance memory. Remarkably, LN γ-1 peptide reversed the PKA and ERK1/2 inhibition induced by anti-PrP^C antibodies (103). These findings show that hippocampal PrP^C plays a critical role in memory processing through interaction with LN and suggest that this role is mediated by PKA and ERK1/2 (103).

The mechanism by which PrP^C-LN interaction modulates memory is still unknown. However, a plausible hypothesis is based on the interaction of LN with the tissue type plasminogen activator (tPA)/plasmin proteolytic cascade. On the one hand, laminins stimulate neurite outgrowth, and the most abundant laminin isoform in the hippocampus is LN-10 (α5β1γ1) (230), which is produced and secreted by neurons. These cells bind to LN-10 through integrin α3β1 (390), as well as through PrP^C (103). The PrP^C-binding domain maps to the COOH-terminal domain of the laminin γ-1 chain, and only PrP^C binds to this domain, through which it is able to promote neurite outgrowth (183). The same LN domain was also shown to promote neurite outgrowth, although the cell surface ligand was ignored (294).

On the other hand, tPA is induced as an immediate-early gene during long-term potentiation (LTP) (434). LTP in the CA1 region of the hippocampus is strongly associated with one-trial (passive) avoidance learning (233, 575; see sect. IV*I*), which is impaired in PrP-null mice. There is a clear correlation between the level of expression of tPA and the intensity of LTP, since mice deficient in tPA have a reduction in LTP (159, 220), whereas tPA overexpressors show enhanced LTP (317). Notably, LN is a substrate of the tPA/plasmin proteolytic cascade (89). It is, therefore, possible that partial LN degradation, consequent to physiological stimuli which elicit enhanced tPA activity, releases the γ-1 chain peptide, which then binds to PrP^C initiating signal transduction. This hypothetical chain of events may be a major component of the PrP^C/LN-mediated memory effects described above.

In turn, the binding of hop/STI1 to PrP^C largely induced retention of STM and consolidation of LTM, acting on the CA1 region of the hippocampus (104). The inhibition of this interaction by intrahippocampal infusion of antibodies against PrP^C or STI1 binding sites disrupted both STM and LTM of one-trial avoidance. Furthermore, infusion of PrP^C peptide 106–126, which competes for PrP^C-STI1 interaction, also inhibited memory consolidation. This effect was not due to peptide toxicity, since memory retention was completely restored 24 h after treatment. Strikingly, the peptide $hop/STI1_{230–245}$, which contains the PrP^C binding site, had a potent enhancing effect in memory retention, which was counteracted by peptide $PrP^C_{106–126}$. Taken together, these results demonstrate that interaction of PrP^C with hop/STI1 is critical to consolidate both STM and LTM and suggest a potential use of the peptide $hop/ST11_{230–245}$ as a therapeutic tool (104).

Thus at least two definite molecular interactions of PrP^C with hippocampal cell surface proteins, hop/STI1 and LN, can mediate effects of PrP^C on memory consolidation, and it is likely that PrP^C modulates memory retention through both these interactions.

Further support of the hypothesis that PrP^C plays important roles in memory and cognition is found in human subjects. The presence of Val at codon 129 of *PRNP* in at least one allele was associated with worsened cognitive performance in elderly subjects (30, 247), with early cognitive decline (117), and with faster decline of intellectual abilities in Downs syndrome patients (126). Conversely, healthy young adults expressing Met at codon 129 in at least one allele exhibit better LTM than those with Val in this codon, albeit STM was unaffected (409). Thus polymorphisms at codon 129, a site that is highly important for the protein structure (418), seems to be strongly related with cognitive performance.

H. PrP^C and Alzheimer's Disease

Interest in relating prion diseases with Alzheimer's disease (AD) stems mainly from their common association with misfolded peptides (2). Early work identified amyloid precursor-like protein 1 (APLP1) as a putative ligand of PrP^C (586), but to date this has not been confirmed. Other possible links relate to the fact that both PrP^C and the APLP1 close relative amyloid precursor protein (APP) bind copper (49, 83) and share common mechanisms of proteolytic processing (98, 209), although the significance of these findings for physiological functions is obscure.

Neurological studies provided evidence of linkage of variant alleles of *PRNP* with AD. The data refer to possible correlations of the polymorphism at codon 129 with either the risk of AD or the cognitive decline of these patients.

No association between the *PRNP* 129 polymorphism and the risk of AD was reported in sporadic AD patients from Spain, Italy, or Japan (76, 110, 127, 398). In contrast, Dutch (129) and Polish (176) patients presenting the 129 allele in homozygosis, particularly 129VV, had a higher risk of early-onset AD when compared with heterozygous. The same was found for German 129MM carriers (446).

The conflicting results may be due to a strong effect of ethnicity on a link between *PRNP* polymorphisms and genetic susceptibility to AD (127). Indeed, meta-analysis of the studies above indicated that Caucasian subjects homozygous at codon 129 had a 1.3-fold increased risk of developing AD, compared with heterozygous. In addition, AD patients carrying at least one V allele presented a small acceleration in their cognitive decline compared with MM carriers (76, 127).

Brains from patients with either AD (142, 548) or Downs syndrome (351) showed an accumulation of PrP^C as globular deposits, which also stained with antibodies to Aβ and to SOD1, suggesting that PrP^C and SOD1 accumulate at Aβ plaques (351). This could result from a response of PrP^C to oxidative stress induced by the Aβ plaque. Since Val at codon 129 is both linked to enhanced cognitive decline (see above) and correlates with the presence and density of Aβ-associated lesions (29), it is possible that neuroprotective effects mediated by PrP^C are less effective when the protein has a Val at codon 129 (351).

Unfortunately, the reports of human subjects are too preliminary to establish a causal chain among the expression of PrP^C, the polymorphism in codon 129, neuroprotection, and memory-associated synaptic plasticity (see Ref. 233). However, investigation of both the proteolytic processing and the properties of distinct PrP^C polymorphisms, as well as their possible links with amyloid precursor protein are warranted.

In fact, recent studies demonstrated that expression of PrP^C inhibits the cleavage of APP by β-secretase (BACE1), thereby reducing the formation of the amyloid β-peptides, the major constituents of the senile plaque (411). This effect was abolished in PrP^C molecules lacking the NH_2-terminal KKRP (23–26) residues, previously associated with PrP^C endocytosis (518). In addition, modifications of PrP^C that cause its exclusion from cholesterol lipid rafts, such as tethering the PrP^C NH_2 terminus to the membrane, deletion of the GPI anchor signal peptide or anchoring PrP^C by a transmembrane domain, also blocked the inhibitory effect of PrP^C on β-secretase activity. These results suggest that PrP^C endocytosis mediated by the KKRP domain, and its localization within specific membrane domains, are necessary for the inhibitory effect of PrP^C upon β-secretase. Nonetheless, two PrP^C mutants associated with familial forms of human prion diseases, PG14 with an extra nine copies of the octarepeat and A116V (equivalent to human A117V), also lost the ability to modulate β-secretase. Since there is no indication that these two mutants are excluded from cholesterol lipid rafts, nor that they have altered endocytosis, additional factors may contribute to the effect of PrP^C on β-secretase activity. Moreover, scrapie-infected mice presented higher β-secretase activity when compared with uninfected animals, indicating that PrP^C loses its normal function on the modulation of β-secretase activity either when holding specific mutations associated with prion diseases or when it is converted to PrP^{Sc} (411). This is important, because mice infected with PrP^{Sc} presented much higher levels of $A\beta_{1-42}$, the peptide associated with AD. It would be interesting to infect APP knockout mice with PrP^{Sc}, to test whether the increased production of $A\beta_{1-42}$ may have a role in the cognitive decline in prion diseases.

In brains from PrP-null mice (Edbg), the levels of both peptides $A\beta_{1-42}$ and $A\beta_{1-40}$ were significantly higher than in wild-type animals, consistent with the proposed inhibitory role of PrP^C on β-secretase activity (411). On the other hand, a study using a double transgenic mouse expressing both mutant amyloid precursor protein and

overexpressed Syrian hamster PrP^C indicated that PrP^C enhances the formation of amyloid plaques without causing any significant alteration in the levels of either $A\beta_{1-42}$ or $A\beta_{1-40}$ (482). These mice expressed endogenous PrP^C and presumably the maximum inhibition of β-secretase activity, although this has not yet been tested in Tg20 mice that overexpress PrP^C. It has, nonetheless, been suggested that overexpression of hamster PrP^C does not cause any further β-secretase inhibition, whereas it is possible that PrP^C has a secondary effect on Aβ aggregation (411).

The effect of PrP^C polymorphisms at codon 129 on β-secretase activity was also addressed in mice whose endogenous *Prnp* was replaced by human *PRNP*. The amount of the peptide $A\beta_{1-42}$ was similar between MM or VV homozygous mice, while peptide $A\beta_{1-40}$ tended to be slightly more abundant in MM mice compared with VV animals (411). The relevance of these data for possible effects of PrP^C polymorphisms on AD await further studies, particularly in view of the evidence for a small acceleration in the cognitive decline of patients carrying the VV polymorphism at codon 129, when compared with MM carriers (76, 127).

The data from Parkin et al. (411) raise important questions related to AD and suggest that modulation of the role of PrP^C upon β-secretase activity may provide a novel therapeutic approach for AD (411). Furthermore, these results add to the evidence for physiological roles of PrP^C (as discussed in the present review), in cautioning against interventions in TSEs that focus on either the blockade or the inactivation of PrP^C.

I. PrP^C, Synaptic Activity, and Neuronal Excitability

The evidence for significant roles of PrP^C in a variety of behavioral processes, and particularly in memory, raises the question of whether and how does the prion protein affect synaptic mechanisms and neuronal excitability.

The presence of PrP^C in both pre- (202) and postsynaptic structures (191) raised the hypothesis that PrP^C plays a role in neuronal communication. In addition, some PrP^C glycoforms can be selectively transported along axons, suggesting that these glycoforms may be specifically presynaptic (450).

A functional role of PrP^C in synapses was suggested on the basis of the ability of PrP^C to bind copper (see sect. II*E*), because nerve endings release copper into the synaptic cleft upon depolarization (252). It was proposed that presynaptic PrP^C may buffer Cu^{2+} levels in the synaptic cleft and ensure its transport back into the presynaptic cytosol. This would also protect synapses from the ROS generated by Fenton-type redox reactions. In addition, the buffering of Cu^{2+} by PrP^C could play a role in calcium homeostasis, because copper, at the concentrations found in the synaptic cleft, reduces Ca^{2+} influx through voltage-gated calcium channels (VGCC) (542).

LTP has long been associated with learning and memory. Recently, it was shown that LTP in the hippocampal CA1 area is at the root of memory formation of one-trial inhibitory (passive) avoidance in the rat (233, 575). Synaptic plastic changes in the basolateral amygdala, the entorhinal, parietal and cingulate cortex, and probably others, are also required for memory consolidation of this task (233). However, CA1 LTP clearly stands out as one crucial component of memory consolidation (575). LTP in other hippocampal subregions, such as the dentate gyrus, or in extrahippocampal brain structures, has frequently been ascribed roles in learning, but their molecular basis is distinct from that of CA1 LTP (325), and evidence for their role in learning is not as compelling as that of the role of CA1 LTP in one-trial avoidance.

In early studies, impaired LTP was found in tissue slices from PrP-null mice, when compared with WT at physiological temperature (108, 332, 577). This was associated with reduced $GABA_A$ receptor-mediated fast inhibition (108) and was rescued by a transgene encoding PrP^C (577). Differing from these data, no deficits were found in cell excitability, synaptic inhibition, reversal potential for inhibitory postsynaptic potentials or LTP, when hippocampal slices from *Prnp*$^{0/0}$ Zrch I animals were examined at room temperature (302). Also, outside-out membrane patches of cerebellar Purkinje cells from these PrP-null mice displayed no change of $GABA_A$ receptors (200). Recently, however, it was shown that the levels of both posttetanic potentiation and LTP in the CA1 region of aged PrP-null mice were significantly reduced, when compared with younger animals, a result that was attributed to increased levels of oxidative stress in aged animals (118).

Contrasting with the impaired CA1 LTP in PrP-null mice, hippocampal slices from ZrchI animals demonstrated increased excitability of the dentate gyrus (323). Thus physiological effects of PrP^C in the dentate gyrus may differ from those in CA1. Still, these data do not challenge the hypothesis that altered LTP underlies the memory loss observed in PrP-null animals. First, dentate gyrus LTP obeys mechanisms very different from those of CA1 LTP. Second, the latter, but not the former, is unequivocally linked to memory formation processes (233, 575).

PrP-null mouse neurons reportedly had lower input resistances but lacked a late I_{AHP}, which may have opposing consequences on neuronal excitability (107). Remarkably, ectonucleotidase activity is diminished in synaptosomes from PrP-null animals, when compared with WT, thus attenuating the hydrolysis of ADP and decreasing the synaptic levels of the anticonvulsant neu-

rotransmitter adenosine (416). Moreover, although synaptosomes from PrP-null animals show normal glutamate uptake (530), this function is impaired in cortical astrocytes (50), which may lead to increased glutamate concentration at the synaptic cleft and higher neuronal excitability. Thus the loss of PrP^C may have either positive or negative effects on neuronal excitability and synaptic transmission, probably as a function of the affected region of the brain, as well as the type of response and intervening neurotransmitter systems elicited by diverse patterns of stimulation.

The afterhyperpolarization potentials (I_{AHP}) depend on calcium influx through L-type VGCC. Interestingly, Ca^{2+} homeostasis is altered in cells from PrP-null animals (199), and patch-clamp studies of cerebellar Purkinje cells showed a significant correlation between PrP^C expression and the maximal amplitude of Ca^{2+}-activated K^+ currents. However, the absence of PrP^C reportedly did not alter the K^+ channels directly responsible for the slow I_{AHP} observed in neurons from PrP-null mice, but rather modulates K^+ channels indirectly by reducing Ca^{2+} influx through L-type VGCC channels (161).

Conditional knockouts, in which the expression of PrP^C is abolished at 12 wk of age, also presented a slow afterhyperpolarization in hippocampal CA1 cells, and thus increased neuronal excitability. Because PrP^C was deleted only in adult life, this phenotype was caused by neuronal dysfunction, rather than by a developmental deficit (328). In keeping with this observation, the impairment of Ca^{2+}-activated K^+ currents previously found in PrP-null mice was rescued in mice where PrP^C had been reintroduced (201). Interestingly, a transgene encoding a mutated PrP^C at codon 200, which is the most common mutation associated with inherited prion disease, was also able to rescue the normal I_{AHP} (13), indicating that this particular protein domain is not responsible for sustaining normal neuronal excitability.

Increased neuronal excitability in PrP-null hippocampus is also consistent with the lower threshold found in ZrchI mice, compared with WT, to seizures induced by either a single convulsant or repeated subconvulsant (kindling) doses of pentylenetetrazol, and to status epilepticus induced by kainic acid or pilocarpine (553). Similarly, both Npu and conditional postnatal PrP^C knockouts also presented a lower seizure threshold than WT (M. C. Landenberger, C. Fagundes, R. Walz, B. Chesebro, G. Mallucci, and V. R. Martins, unpublished results). The expression of a single *Prnp* allele in heterozygous animals was sufficient to rescue the normal phenotype. Notably, Tga20 mice, which express 10 times more PrP^C than WT (146), are highly resistant to seizures induced by either kainic acid or pentylenetetrazol (Landenberger et al., unpublished results). These data demonstrate that expression of PrP^C is directly correlated with a higher resistance to seizures.

The higher excitability of PrP-null mice is also consistent with anatomical observations, specifically of the mossy fiber pathway within the hippocampus. Timm-stained brain slices from PrP-null mice exhibited more granules than controls in the granule cell layer, the inner molecular layer of the dentate gyrus, and the infrapyramidal region of CA3 (107). The observed reorganization of neuronal circuitry is similar to the mossy fiber collateral and terminal sprouting seen in temporal lobe epilepsy related to hippocampal sclerosis and may represent an "epileptic neuronal network."

Thus the combined data demonstrate that PrP^C significantly modulates neuronal excitability and synaptic activity, which likely constitute the neural basis for at least some of the systemic brain functions attributed to PrP^C.

V. IMMUNOMODULATORY FUNCTIONS OF THE PRION PROTEIN

The main thrust of research in prion biology is, understandably, aimed at the CNS. However, expression of PrP^C is widespread, selectively enriched, and developmentally regulated in certain cell types also outside the nervous system (see sect. II*C*).

Despite the lack of both overt peripheral inflammatory signals, and of antibodies against the PrP^C in the course of TSE, substantial amplification of the infectious agent takes place within lymphoid compartments in early stages of the diseases. In addition, both the state of the immune system and conditions that challenge innate immune defenses affect the susceptibility and the course of experimental prion infection (4, 111). Similar to the CNS, PrP^C in immune cells has been usually studied as far as either pathogenesis or treatment are concerned. Here we examine evidence for immunomodulatory functions of PrP^C in physiological context.

A. Evidence for a Role of PrP^C in the Immune System

No gross defects were found in PrP-null mice in either numbers or maturation of cell components of the immune system, including hematopoietic stem cells, nor in the expression of critical cell surface antigens (see Ref. 232 for review). This suggests that immunomodulatory functions of PrP^C are either subtle, or may be relevant only in particular contexts. Indeed, it was argued that expression of PrP^C in hematopoietic progenitors and mitotic lymphocytes might be due to high rates of membrane turnover or cell division (151).

A preliminary caveat is that the expression of PrP^C is nonhomogeneous both across species and among subsets and states of maturation of immune cells (Table 1). As a

rule, the expression of PrP^C is downregulated with maturation of granulocytes and upregulated with maturation of myeloid antigen-presenting cells (APC). However, among lymphoid cells, maturation was accompanied by downregulation of PrP^C compared with precursor cells in mice, but apparently upregulated, or at least maintained at elevated levels in mature human lymphoid cells (see references in Ref. 232).

PrP^C affects the ability of long-term hematopoietic stem cells (HSC) to sustain self-renewal under stressful conditions (597). It was not resolved whether proliferation or, alternatively, resistance to cell death, underlies the enhanced self-renewal ability of HSC to reconstitute bone marrow. Nonetheless, these results support the hypothesis that PrP^C modulates the responses of immune cell precursors to extracellular factors and highlight an important role of PrP^C in the immune system. Previous studies had, however, already pointed at this direction.

B. Lymphoid Cells

Early work showed that activation increased PrP^C at the surface of both human lymphocytes and lymphoid cell lines, and anti-PrP^C antibodies prevented concanavalin A (ConA)-induced proliferation (77). Subsequent studies confirmed the upregulation of PrP^C following stimulation with multivalent lectins (19, 289, 315) and showed reduced mitogen-induced proliferation in PrP-null murine splenic lymphocytes (315). Other reports contained conflicting results about differential responses of either WT or PrP-null splenocytes to mitogens (17, 19, 301, 348), but PrP^C transfected into PrP-null murine splenocytes sustained an increased ConA-induced proliferative response, compared with the parental PrP-null splenocytes transfected with an empty plasmid (17).

Interestingly, supernatants of transfected PrP^C-expressing splenocytes contained higher amounts of both IL-2 and IL-4, compared with PrP-null splenocytes following stimulation with ConA, which requires the T-cell receptor (TCR) complex, but not after stimulation with phorbol ester plus ionomycin, which bypasses the TCR (17). Previous work had shown that removal of GPI-anchored proteins prevented T-cell activation by ConA, but not direct activation via the TCR (426, 532). The overall data suggest that PrP^C cooperates with the TCR in the activation of T cells induced by a cross-linking lectin.

Confocal microscopy of CEM-T human lymphoid cells showed colocalization at the level of the plasma membrane, of PrP^C with the Src-family non-receptor tyrosine kinase Fyn, but not Src itself. A polyclonal anti-PrP^C antibody coimmunoprecipitated Fyn, and conversely, anti-Fyn pulled down PrP^C in both resting and CD28/CD3 stimulated cells. Following stimulation, anti-PrP^C also coimmunoprecipitated the Syk family tyrosine kinase ZAP-70, which binds both CD3ζ chains and CD45-associated phosphatase upon TCR stimulation (343). These data support the interpretation that PrP^C associates with the TCR complex and with at least some of the latter's intracellular downstream effectors.

An alternative mechanism linking PrP^C with T-cell function was based on the result that IL-2 production was delayed in PrP-null splenocytes stimulated by ConA in the presence of a Cu^{2+} chelator. The authors suggested that rapid Cu^{2+} transport by PrP^C may be involved in T-cell activation (269), but alternative explanations for the role of Cu^{2+} may apply (see sect. II*E*).

ConA and PHA are tetrameric at physiological pH (358, 554), and therefore, their effects likely rely on lateral reorganization of surface glycoproteins, which may include not only the TCR and CD3, but also PrP^C. The requirement for lateral reorganization is also apparent in other PrP^C-mediated responses. In CEM-T cells, PrP^C antibodies modulated both thapsigargin-induced Ca^{2+} release and entry through store-operated Ca^{2+} channels, with the most pronounced effects occurring upon specific PrP^C cross-linking with either avidin or polyclonal antibodies, which likely promote substantial lateral movement of PrP^C (225). The authors suggested that a physiological ligand associated with PrP^C-mediated T-cell responses would be the most efficient if multivalent (225). However, the effects described may be related to particular experimental conditions, which promote the concentration of PrP^C molecules dragging a particular set of components of a multi-protein cell surface signaling complex (e.g., Ref. 339).

Indeed, cross-linking of PrP^C in Jurkat T cells and peripheral blood T lymphocytes produced both cocapping of PrP^C with the raft-associated proteins reggie-1 and reggie-2 (flotillin-2 and flotillin-1, respectively), as well as a transient Ca^{2+} signal and phosphorylation of Erk1/2, which were somewhat distinct from similar signals induced by TCR/CD3 stimulation. In addition, PrP^C cross-linking also dragged the Src-family kinases Fyn and Lck, as well as CD3 and LAT to the cap, consistent with recruitment of a functional TCR complex, followed by endocytosis of PrP^c together with the reggies (512).

These results show that cross-linked PrP^C can promote the assembly of TCR complex components within rafts, and this dynamic behavior of a set of cell surface, transmembrane, and juxtamembrane molecules may constitute the basis of several PrP^C-mediated T-cell responses described above. Responses induced by the multivalent lectin PHA were selectively blocked with antibodies to certain epitopes in the PrP^C molecule, but not to others (289). This may be due to selectively interfering with the interaction of PrP^C and other cell surface components necessary for the assembly of the TCR complex in specific experimental context.

C. Antigen-Presenting Cells/Mononuclear Phagocytes

Despite the focused interest on APC in prion infectivity (4, 316), few studies have dealt with physiological roles of PrP^C in dendritic cells (DCs) and macrophages. PrP^C is expressed in myeloid cells (19, 66, 135, 151, 240, 301, 337). Increased expression of PrP^C consequent to DC maturation paralleled that of the MHC class II and costimulatory molecules, as well as the production of IL-12 induced by either TLR4 or TLR9 ligands (337). PrP^C also colocalized with MHC class II in monocyte-derived DCs (66). However, PrP-null mice also showed increased expression of MHC class II and costimulatory molecules, upon lipopolysaccharide (LPS)-induced maturation of DCs in vitro (19). Thus PrP^C is not required for maturation of DCs.

Recently, the behavior of interacting DCs and T cells was examined in the context of an allogeneic mixed lymphocyte reaction (MLR) and a peptide-MHC driven proliferative T-cell response (19). Confocal microscopy of T/DC contact points in the latter case showed accumulation of PrP^C at the contact zones, but no colocalization with CD3, LFA-1, or LAT, all of which reside at various points at the immunological synapse, nor with CD43 or CD90/Thy-1. The absence of PrP^C in the DCs abrogated the proliferative T-cell response. Antibodies to PrP^C also prevented T-cell proliferation in both types of response involving WT DCs and either WT or PrP-null T cells. The same was observed in a peptide-MHC driven response in vivo. In contrast, the absence of PrP^C in the T cells did not prevent their APC-induced proliferation, but antibodies to PrP^C blocked the T-cell response in MLR between PrP-null DCs and WT T cells.

These experiments are arguably more representative of physiologically relevant functions of PrP^C than studies employing multivalent lectins and cross-linking antibodies. Furthermore, both the APC and the target T cells were examined in the context of the immunological synapse, including in vivo responses (see Refs. 136, 298 for reviews). Comparing the effects of either intact Ig or Fab fragments of the PrP^C antibody indicated that their effect on the DCs was not due to cross-linking of PrP^C and supports the hypothesis that PrP^C in DCs is a positive regulator of the immunological synapse. In turn, the antibody effect on the T cells, coupled to normal response of PrP-null T cells, suggests that the antibody to PrP^C had an agonist effect on the latter cell type, although it is not clear whether this may depend on cross-linking of PrP^C.

In summary, this study (19) provided strong evidence for an important role of PrP^C upon the interaction of APC and T cells, although its molecular basis is still unclear. Notably, inhibition of T-cell proliferation by intact antibody to PrP^C is consistent with evidence that antibody-mediated mobilization of GPI-anchored proteins on T cells may provide a negative signal for clonal expansion (336, 585), rather than with the assembly of the TCR complex signaling pathways promoted by cross-linking antibodies to PrP^C (512), or the agonist effects of GPI-anchored proteins on T-cell responses suggested by enzymatic removal of the former (426, 532).

As for macrophages, it was reported that PrP^C is required for macrophage invasion by the Gram-negative bacteria *Brucella abortus*, through interaction with bacterially secreted Hsp60 (558, 559). However, these findings have been challenged, because blocking antibodies to PrP^C did not affect macrophage invasion by *Brucella*, there was no difference in the infection of WT or PrP-null macrophages or mice, and no specific binding was demonstrated between *Brucella* and recombinant PrP^C (149). Therefore, a role of PrP^C on the interaction of microorganisms with macrophages (3) is dubious.

In a distinct approach, a soluble PrP^C-Fc fusion protein was applied to the P388D-1 mouse macrophage-like cell line. The fusion protein bound to the cell surface and induced tyrosine phosphorylation, as well as phosphorylation of the Erk and Akt kinases (268). Nonetheless, P388D-1 is a triploid, highly unstable cell line derived from a lymphoblastic pre-B lymphoma, with a high degree of genomic instability and abundant cytogenetic aberrations (106). It is therefore of little value as a surrogate macrophage/monocyte of physiological relevance. Investigation of a more conventional macrophage cell line, or indeed of primary macrophages, may provide more reliable evidence on the possible effects of extrinsic PrP^C on the latter.

We examined the role of PrP^C in the phagocytosis of apoptotic cells by peritoneal macrophages. Macrophages from PrP-null mice exhibited higher rates of phagocytosis in vitro of a variety of apoptotic cell types than WT macrophages. Elimination of GPI-anchored proteins from the cell surface of WT macrophages rendered these cells as efficient as PrP-null macrophages (120).

PrP^C on the macrophage surface seemed enough for the distinct responses, since apoptotic leukocytes derived from WT or PrP-null mice were phagocytosed with similar efficiency, but PrP-null macrophages were more effective in phagocytosing either type of leukocyte (Fig. 6). Furthermore, both the phagocytosis of retinal apoptotic bodies by PrP-null Muller glial cells in situ and of zymosan by peritoneal PrP-null macrophages in vivo were higher than WT (120). These data show that PrP^C is a negative modulator of phagocytosis, which may help balance the threshold of phagocytic responses (see Ref. 121 for review).

D. PrP^C in Inflammatory Responses

Following intraperitoneal injection of zymosan, there was reduced leukocyte infiltration, and few polymorpho-

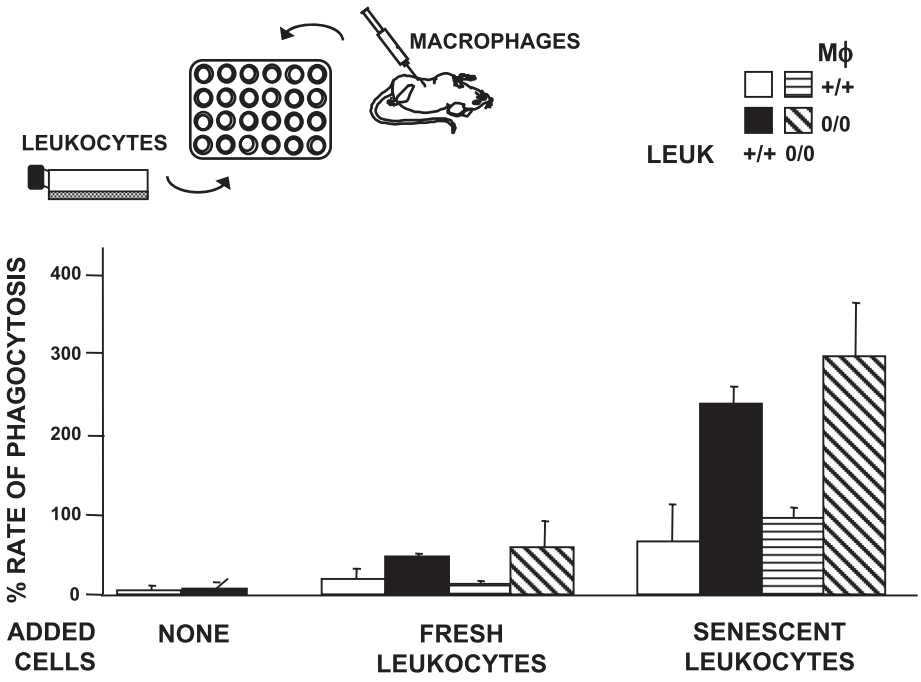

FIG. 6. Role of PrP^C in phagocytosis. Peritoneal leukocytes, either freshly removed or senescent, were seeded on top of preplated peritoneal macrophages, and the rate of phagocytosis was estimated following 60 min of coincubation and washing. The rates of phagocytosis were normalized with respect to phagocytosis of PrP-null apoptotic leukocytes by wild-type macrophages. [Data from de Almeida et al. (120) and C. J. de Almeida and R. Linden, unpublished data.]

nuclear cells were recruited to the peritoneum of PrP-null mice at 6 h after injection, in sharp contrast to the robust PMN infiltrates in WT (120). A similar result followed intraperitoneal injection of LPS (R. Mariante, A. Nobrega, and R. Linden, unpublished results). In addition, preliminary experiments employing a model of Fas-dependent induced pulmonary silicosis (37) showed that PrP-null mice undergo reduced leukocyte infiltration in the bronchoalveolar liquid, reduced body weight loss, and reduced pulmonary fibrosis, compared with WT after intratracheal instillation of silica (Fig. 7).

These data indicate that PrP^C modulates various aspects of peripheral inflammation induced by ligands of TLR2, TLR4, or Fas, but the mechanisms involved in these multiple effects of ablation of PrP^C have yet to be determined.

E. Implications of PrP^C Functions in the Immune System

Despite the involvement of specific immune cell types in the accumulation of PrP^{Sc} in peripheral lymphoid compartments at early stages of prion diseases (see Ref. 4 for review), no attention has been paid to whether PrP^C is depleted in the immune cells and possible consequences for immune responses. The data reviewed above show that PrP^C may play important roles in the development and maintenance of the immune system, as well as in specific cellular immunological responses. Time is ripe for examining possible loss-of-immune-function components of prion diseases, at least in the context of peripheral infection. Moreover, changes in the behavior of immune cells and associated deregulation of cytokines and chemokines may in itself have an impact on neurodegeneration (67, 75).

VI. ROLES OF THE PRION PROTEIN BEYOND THE NERVOUS AND IMMUNE SYSTEMS

Functions of PrP^C outside the nervous and immune systems are still largely unknown, but putative roles were proposed based on data from transgenic mice and expression of PrP^C.

A. Neuromuscular Junction and Muscle

No major abnormalities of neuromuscular function had been described in early work with PrP-null mice (41). However, a forced swimming test, which measures muscle activity and resistance to fatigue, showed impairment of locomotor activity under extreme exercise conditions, thus suggesting a role for PrP^C in muscle physiology (387). Differing from previously reported mitochondrial abnormalities (356), no change in mitochondrial respiration was found in PrP-null mice (303). The finding of functional impairment only under stress (387) is similar to other changes observed in PrP-null mice (217, 334, 386, 501, 562). However, the authors cautioned that the impairment of locomotor activity in the forced swimming test was inferred from behavioral parameters, and not from direct assessment of muscular function. Thus, alterna-

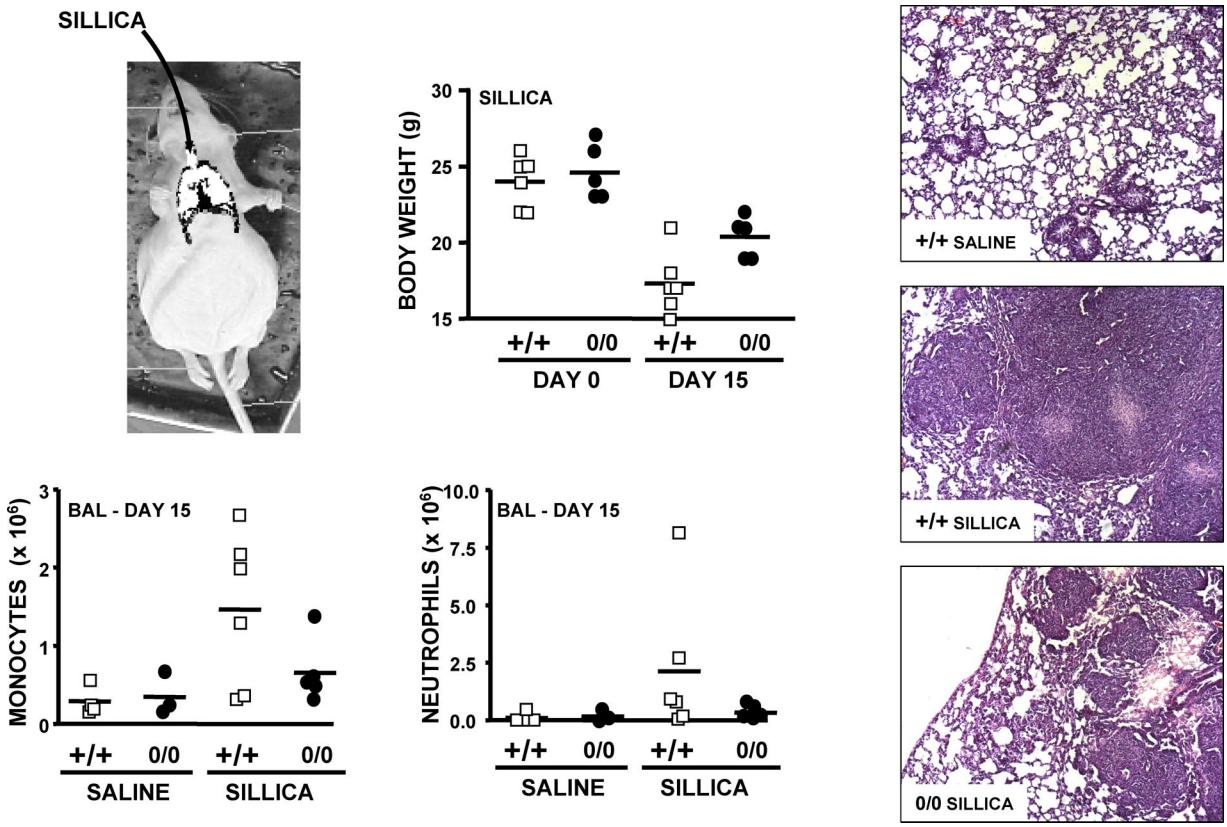

FIG. 7. Role of PrP^C in inflammation. Either wild-type or PrP-null mice received intratracheal injections of sillica, which produces a Fas-dependent inflammatory reaction leading to pulmonary fibrosis (37). The animals were examined at 15 days after the injections. PrP-null mice lost less body weight, and their bronchoalveolar lavage fluid (BAL) contained fewer inflammatory cells than wild type. Histopathological analysis of pulmonary tissue showed reduced lung fibrosis in PrP-null mice, when compared with wild type. (Data from C. J. de Almeida, V. M. Borges, P. R. M. Rocco, C. Takyia, G. A. dos Reis, and R. Linden, unpublished data.)

tively, these results might be an indication of modifications in either motor coordination or in anxiety levels, leading to less effective swimming and earlier fatigue (386).

Older mice overexpressing the *Prnp*^{b} allele suffered from a profound, gene dose-dependent, necrotizing myopathy of skeletal muscle, but not cardiac or smooth muscle. Little PrP^{res}, probably representing aggregation, was accumulated, but PrP^{res} did not associate with onset of disease. Therefore, the myopathy is a consequence of overexpression of PrP^C, and not of PrP^{res} (570). Conversely, transgenic mice overexpressing the *Prnp*^{a} allele presented no noticeable myopathy (146), although muscular function was not assessed.

The PrP alleles (*Prnp*^{a} and *Prnp*^{b}) described above differ at codons 108 and 189 (see sect. IV*D*). It is not known whether these amino acid changes affect PrP^C function, in particular when the protein is overexpressed. However, if the myopathies observed in mice expressing *Prnp*^{b} are in fact related to altered PrP^C function, then the frequency and risk of *PRNP* polymorphisms in human myopathies warrant further investigation.

Expression of PrP^C is increased in sporadic and hereditary inclusion body myositis and myopathy, polydermatomyositis, and neurogenic muscle atrophy (15, 592). Moreover, a uniform pattern of increased PrP^C expression was described in a series of muscular disorders (267). The PrP was proteinase-K sensitive, which rules out PrP^{Sc} toxicity. Interestingly, both glycoform profile and size of PrP^C in normal muscle are distinct from human brain (592).

Based on these findings, it was suggested that PrP^C may have a general stress-response effect in neuromuscular disorders (267). This hypothesis is supported by the accumulation of PrP^C in muscle fibers of an experimental model of chloroquine-induced myopathy (162).

In addition, PrP^C was upregulated when myotubes differentiate from immortalized C2C1 murine myoblasts (53). PrP^C content progressively increased during maturation of myocytes in primary cultures of skeletal muscle, attributed to both transcriptional and posttranslational changes. Fast muscle fibers present a higher concentration of PrP^C than slow fibers, also consistent with a role of PrP^C in skeletal muscle physiology (340).

A severe dilated cardiomyopathy has also been described in a patient diagnosed as sporadic CJD, and a heart biopsy contained evidence of the presence of PrP^{Sc}. Since no other cause was found, it was suggested that the disease derived from the accumulation of PrP^{Sc} into the heart (14). However, the possibility of PrP^{C} loss of function cannot be excluded (31). Recently, disease-associated PrP was also detected in cardiac myocytes of elk and white-tailed deer infected with chronic wasting disease (CWD), but heart physiology was not evaluated (241).

These data raise the hypothesis that PrP^{C} may have important functions in both skeletal and cardiac muscle. Analysis of functional parameters, such as contractile function and neuromuscular junction properties, in both constitutive and conditional PrP-null mice and muscle cells, and particularly under conditions of stress (386), should help clarify this issue.

B. Liver

PrP^{C} is barely detectable in the liver (see sect. II*C*), but increased levels of both PrP^{C} mRNA and protein were detected during hepatic stellate cell activation (228). These cells undergo activation and proliferation under stress, a process that is closely related to liver fibrosis (160). PrP^{C} is particularly detected in the fibrous septa of carbon tetrachloride-damaged liver and in sinusoidal linings of common bile duct-ligated liver. Thus it was suggested that PrP^{C} may be involved in the response to oxidative stress and in the development of liver fibrosis (228).

C. Pituitary

Functions of PrP^{C} in neuroendocrine secretion were addressed in melanotrope cells of the intermediate pituitary of *Xenopus laevis* (538). When animals are exposed to a dark background, these cells produce high levels of proopiomelanocortin (POMC) (239). POMC is processed into several bioactive peptides, including α-melanophore-stimulating hormone (α-MSH), the release of which causes melanin dispersion and darkening of the skin (239, 454). Thus this model allows the physiological manipulation of biosynthetic and secretory activities of melanotrope cells.

PrP^{C} was detected both throughout embryonic development and in adult frogs, but its expression was not affected during background adaptation, indicating that it does not affect POMC biosynthesis (538). *Xenopus* transgenesis was used to overexpress PrP^{C} under control of the POMC promoter specifically in the melanotrope cells. Subcellular structure, POMC synthesis and processing, and the secretion of POMC-derived products remained unaltered within the PrP^{C}-overexpressing cells (538, 539). However, secretion of POMC was reduced when cells expressed PrP^{C} for long periods (540). Remarkably, electron microscopy revealed the induction of lysosomes taking up POMC secretory granules (crinophagy), which is probably responsible for the reduced POMC levels. Thus, in neuroendocrine cells from *Xenopus*, PrP^{C} modulates the secretory pathways inducing crinophagy (540).

Functional properties of PrP^{C} in mammalian pituitary have not been addressed to date, but its role in POMC secretion deserves further investigation, due to the importance of the melanocortin system particularly in the regulation of feeding behavior and weight homeostasis.

VII. ROLES OF THE PRION PROTEIN ON THE CELL LIFE CYCLE

Functions subserved by PrP^{C} clearly outreach the nervous system. Indeed, abundant data are available on the roles of PrP^{C} in homeostatic processes irrespective of cell type.

Early embryonic and progressive expression of PrP^{C} mRNA and/or protein is generally interpreted as evidence for developmental roles of PrP^{C} (279, 293, 330, 349, 355, 467). During embryogenesis, as well as in continually renewing cell populations of the adult organism, developmentally regulated molecules may be involved in mechanisms related to cell proliferation, differentiation, and programmed cell death. Each of these cellular responses is reviewed in turn.

A. PrP^{C} and Cell Proliferation

Expression of PrP^{C} also correlated positively with the rate of proliferation in both the subventricular zone and the dentate gyrus in adult mice. There were no morphometric differences among the brains of mice containing no, normal, or overexpressed PrP^{C} (506), indicating that the PrP^{C}-dependent increased cell production is compensated by other events to define the final cellular composition of the brain (578). Interestingly, however, PrP^{C} in the proliferating regions of the adult brain was found restricted to postmitotic neurons (506). This suggests an indirect effect on the proliferation of the underlying mitotic precursors, a possibility that must be entertained when dealing with complex tissues.

Nonetheless, the expression of PrP^{C} at the surface of certain cells confers higher rates of mitogen-induced proliferation (315, 348), which may be due to enhanced sensitivity to growth factors present in the cellular microenvironment, as well as changes in the downstream cell cycle machinery.

Based on an array of 597 mouse DNA clones, several cell cycle-related genes, such as cyclin D1, Eps8, and CD44, were reportedly downregulated, whereas certain

other genes associated with growth factor-related signal transduction were upregulated in PrP-null compared with WT cells (473). In contrast, Northern blots of brain extracts showed a substantially lower level of expression of both Eps8 and CD44 in PrP-null, when compared with WT, whereas cyclin D1 displayed no difference, and the expression of the growth-factor related genes was similar in both WT and PrP-null brain extracts (473). It is, therefore, possible that effects of altered *Prnp* gene expression on the levels of expression of cell cycle-related genes may contribute to the control of cell proliferation by PrP^C in a cell type-specific way.

B. PrP^C and Cell Differentiation

Data on this subject are somewhat contradictory. For example, both PrP^C mRNA and protein were downregulated along progressive differentiation of granulocytes derived either from bone marrow precursors or from retinoic acid-treated HL60 leukemia cells (132). In contrast, PrP^C mRNA was upregulated upon both growth arrest and terminal differentiation of murine erythroleukemia cells, as well as human neuroectodermal and myoid cell lines (181).

Studies of the expression of PrP^C in developing neurons also provided conflicting results. In paraformaldehyde-fixed sections of developing hamster brain, monoclonal antibody 3F4 initially labeled mainly axons. Then, at later stages, staining shifted to synaptic beds, suggesting that PrP^C is involved in axon growth or guidance, followed by a role in synaptogenesis, both of which are major differentiation events of neuronogenesis (467). In cultures of multipotent neural precursors, PrP^C content positively correlated with differentiation, as judged by reduced nestin and increased MAP2 (506).

In contrast, PrP^C was equally distributed in both dendrites and axons of cultured hippocampal neurons, mostly within intracellular compartments in early stages. Then, PrP^C evolved to a restricted distribution at the axonal surface, allegedly at stages when axon growth has stopped, with no colocalization with the synaptic marker synaptophysin (166). These controversial findings highlight the difficulties in attributing function to PrP^C on the basis of its selective distribution alone. Stronger evidence arises from comparative studies of either mice or cells expressing variable amounts of PrP^C, as well as on experimental studies of the effects of soluble PrP^C and its ligands on cell differentiation.

Early work had shown that the expression of PrP^C affects morphological differentiation of hippocampal neurons in vitro. Thus both at short-term and in response to stimulation with a phorbol ester, neurons derived from Ngsk PrP-null mice grew shorter neurites than WT neurons, which was reverted by transfection of the *Prnp* gene (274). In recent work, the acquisition of MAP2 also correlated with PrP^C content among neural precursors of WT, knockout, and overexpressing mice (506).

Recombinant PrP^C binds to both the hippocampal neuropil and to cerebellar granule cells in histoblots of WT, PrP-null, and PrP^C-overexpressing mouse brain (284), as well as to primary cultures of neurons from either WT or PrP-null mice (171). Cultured granule cells from the mouse cerebellum treated with either a substrate of PrP^C-Fc or a feeding layer of PrP^C-expressing, transfected CHO cells, responded with neurite outgrowth, which also occurred with mouse embryonic hippocampal neurons treated with PrP^C-Fc. Both WT and PrP-null neurons were equally sensitive to the effect of PrP^C (86, 471).

Further evidence that *trans*-acting PrP^C induces neurite outgrowth was obtained in cultures of neurons derived from the hippocampus of rat embryos. Soluble, recombinant PrP^C induced vigorous neurite outgrowth, both dendritelike and, particularly, a robust single axonlike process per neuron, and enhanced synaptogenesis, recognized as overlapping staining for both synaptophysin and the postsynaptic protein PSD-95, among a richly interweaving network of processes (249). Interestingly, immunostaining of the exogenous protein applied to the cultures indicated that the neurite-promoting effect was not associated with internalization of PrP^C.

These data strongly indicate that the binding of PrP^C to target cells triggers neuronal differentiation. Notwithstanding the possibility that either NCAM or the laminin receptor precursor may underlie these effects, the true cell surface receptor complex involved in the transduction of the PrP^C-induced differentiation signals remains unidentified.

In turn, identified ligands of PrP^C have been shown to induce PrP^C-dependent neuronal differentiation. This is the case for laminin, the cochaperone hop/STI1, and vitronectin.

Process outgrowth from either NGF-primed PC12 cells or mouse hippocampal neurons was selectively blocked by either antibodies to PrP^C or by chromophore-assisted laser inactivation of cell surface PrP^C. In addition, laser ablation of PrP^C led to retraction of outgrown laminin-induced processes in PC12, and laminin-induced differentiation was abrogated in hippocampal neurons from $Prnp^{0/0}$ mice (183, 184). The PrP^C-mediated effect of laminin is selectively induced by the LN γ-1 peptide, which contains the PrP^C-binding site in LN (see sect. II*D*). Two important pending issues are *1*) whether PrP^C-sensitive morphological differentiation is strictly an adhesion effect of laminin (184), and *2*) whether neurite outgrowth induced by the PrP^C-laminin interaction is accompanied by phenotypic differentiation into either dendrites or axons, such as demonstrated for soluble PrP^C (249).

Similarly, the recombinant cochaperone hop/STI1 induced neuritogenesis in cultured hippocampal neurons

from WT, but not from *Prnp*$^{0/0}$ mouse embryos, an effect that was abolished by antibodies against either PrP^C or hop/STI1 and was mimicked by a peptide corresponding to the hop/STI1 domain (aa 230–245) that binds PrP^C (306).

Finally, vitronectin, another component of the extracellular matrix, promoted axonal growth in dorsal root ganglion neurons (DRG) from WT mouse embryos, an effect that was mimicked by vitronectin peptide 307–320, which contains the PrP^C binding site. In DRGs from two distinct PrP^C-null mice strains, vitronectin, but not vitronectin peptide 307–320, induced axonal growth. Functional assays demonstrated that PrP^C-null DRG neurons are more responsive than WT neurons to Arg-Gly-Asp peptide, which is the vitronectin binding site for integrins. PrP^C-null DRG also presented a higher activity of the integrin $\alpha_v\beta_3$ (192). This, together with changes in the basal activity of several signal transduction pathways demonstrated in PrP-null mice (see sect. VIII, *C* and *D*), provides evidence for compensatory mechanisms upon constitutive abrogation of PrP^C.

In summary, the overall data show that both PrP^C and at least some of its ligands can induce neuronal differentiation and suggest that PrP^C may also mediate the differentiation of nonneuronal cells.

C. PrP^C in Cell Death and Survival

Early evidence that expression of PrP^C modulates the sensitivity to cell death was obtained in cell lines established from hippocampal neurons derived from either WT or Ngsk PrP-null mice by retroviral-mediated transfection of the simian virus 40 (SV40) large-T antigen. PrP-null cells died quickly upon serum deprivation, whereas their WT counterparts survived. Resistance to cell death was conferred by transfection of knockout cells with *Prnp*, similar to the effect of *Bcl-2* transfection (274). In either WT or mock-transfected cells, serum deprivation led to p53 and Bax upregulation, cleavage of both caspase-3 and poly(ADP-ribose) polymerase (PARP), decreased Bcl-2 and mitochondrial cytochrome *c*, increased levels of mitochondrial calcium, and lowered mitochondrial membrane potential. In contrast, transfection of knockout cells with a *Prnp* expression vector prevented all those typical apoptotic changes (259).

In cultured human fetal neurons microinjected with a variety of cDNAs, apoptosis induced by injection of *Bax* was prevented by coinjection of *Prnp*, similar to the well-known protective effect of *Bcl-2*. *Prnp* antisense treatment did not by itself induce cell death, but enhanced the degenerative effect of *Bax*. Interestingly, whereas interfering with the traffic of secreted proteins along the Golgi abolished the protection conferred by *Prnp* microinjection, expression of a soluble form of PrP^C lacking the GPI anchor was as effective as regular GPI-anchored PrP^C (39). The latter result is consistent with the increased survival conferred by *trans*-interacting PrP^C upon both cerebellar granule cells and mouse hippocampal neurons (86, 249). A protective effect was also demonstrated for cytosolic PrP^C (456).

Ectopic expression of mammalian *Prnp* in yeast also prevented Bax-induced cell death (286). However, whereas deletion of the octapeptide repeats abrogated the protective effect of PrP^C in human neurons (39), it did not affect yeast. Also contrary to human neurons, cytosolic PrP^C failed to provide protection against Bax-induced cell death in yeast (286). It should be noticed that cell death associated with prion diseases may not be restricted to a single Bax-dependent mode, among a variety of available cell death pathways (190). For example, evidence was provided that cell death associated with NH_2-terminal truncated forms of PrP^C may be either Bax dependent or Bax independent (285).

Analysis of human neurons, mouse neuronal cell lines, and MCF7 breast cancer cells showed that PrP^C delays the conformational change of Bax required for inducing the mitochondrial release of cytochrome *c*. Bax-induced events downstream of mitochondria were unaffected by PrP^C, and experiments with isolated mitochondria suggested that PrP^C does not interact directly with Bax. In addition, PrP^C did not affect cell death induced by either Bak or truncated Bid, nor by staurosporine or thapsigargin in MCF7 cells (455).

It has also been reported that PrP^C protects against ethanol-induced, Bax-mediated brain cell death in vivo. However, the results were not consistently related to the level of expression of PrP^C, and the differences reported were not statistically significant (164). Transient protection of axotomized facial motoneurons was reported for PrP^C overexpressing mice. Nonetheless, motoneuron loss in both WT and PrP-null mice was similar, and there were no differences among the three genotypes following facial nerve transection in adult mice (115).

Other studies, however, provided stronger evidence that the expression of PrP^C has a neuroprotective role upon hypoxic-ischemic insult to the brain in vivo. Thus PrP^C was upregulated in the infarcted brain within a day of permanent focal cerebral ischemia in mice (350, 561), as well as within the penumbra region following transient focal ischemia in rats (492). Increased numbers of PrP^C-immunoreactive profiles were found both at the penumbra of hypoxic damage in adult human brain and in cases of perinatal hypoxic-ischemic injury, accompanied by increased in situ hybridization signals (350). Brains of PrP-null mice suffered more extensive damage than WT following both permanent and transient ischemia (466, 562), and a gene dose-response, protective effect was observed among WT, homozygous, and heterozygous PrP-null mice (350, 501). However, PrP^C above normal levels did not

provide additional protection in the cerebral cortex, and the rate of cell death within the striatum was the same among WT, knockout, and overexpressing mice (501). Finally, overexpression of PrP^C following intracerebral transduction of an adenoviral vector reduced infarct volume and improved neurobehavioral signals after cerebral ischemia in rats (492).

PrP^C also protects against apoptosis in cancer cells. Samples of gastric adenocarcinoma contained increased expression of PrP^C, which correlated with both histopathological differentiation and tumor progression. Modulation of the expression of PrP^C in a gastric cancer cell line confirmed that PrP^C blocked apoptosis, accompanied by upregulation of Bcl-2 and downregulation of both p53 and Bax (291). Also, the expression of both PrP^C mRNA and protein is severalfold higher in TNF-resistant than in TNF-sensitive MCF7 breast carcinoma cells. Consistently, gene transfer of PrP^C in both TNF-sensitive MCF7 and BT20 cell lines conferred resistance to TNF-induced cell death (130).

In sharp contrast to the data supporting a protective role for PrP^C, transgenic overexpression of PrP^C led to a gene dose-dependent, unprovoked neurodegenerative phenotype (570). In line with these results, ectopic expression of PrP^C potentiated staurosporine-stimulated, caspase 3-dependent apoptotic cell death in both the HEK293 and in an inducible PrP^C-transfected cell line derived from rabbit kidney epithelial cells. The proapoptotic effect of PrP^C was potentiated by proteasome inhibitors and abrogated by sequestration of cell surface PrP^C with antibodies (400).

Work was extended (401, 402) with the use of a cell line derived by sequential oncogenic retroviral infection of neocortical neuronal precursors with SV40 large-T antigen, followed by simultaneous v-Src and v-Myc (97), HEK293, and cultured neurons derived from WT and *Prnp*−/− mice. Both overexpression of PrP^C in the cell lines as well as its endogenous expression in WT neurons, compared with knockouts, enhanced basal and staurosporine-induced toxicity and caspase-3 activation by staurosporine, ceramide, and etoposide, an effect dependent on p53. Conversely, *Prnp* antisense diminished staurosporine-induced apoptosis (401, 402).

Notwithstanding, increased cellular content of PrP^C in MCF7 cells did not affect staurosporine-induced cell death (455), the rates of either basal or anisomycin-induced cell death were similar in retinal explants from either WT or $Prnp^{0/0}$ mice (92, 591), and both basal and the increased survival induced by soluble PrP^C were indistinguishable between WT and PrP-null cerebellar granule neurons (86). Thus conditions for either cytoprotective or proapoptotic effects of modulating the expression of PrP^C may vary among distinct cells and tissues.

Certain experimental preparations offer distinct operational advantages, which may be, nonetheless, accompanied by critical caveats. Immortalized cell lines express disregulated genes directly or indirectly associated with mechanisms of cell death, some of which may either coincide with or affect essential intermediaries of PrP^C-associated effects. For example, a critical interplay of the oncogenes *src* and *myc* affects the transactivation of p53-regulated genes in the context of cell death (545), which might affect the p53-dependent degenerative effects of PrP^C in a *v-src/v-myc* transformed cell line (97, 402). Similar considerations may apply to the HEK293 cell line, originally derived by adenoviral-induced transformation (182), which shows a rather distinctive gene expression profile, similar to other transformed cell lines, and quite different from nontransformed human embryonic kidney and other cells (251), which in turn may affect other studies of PrP^C-modulated cell death (401, 402). The role played by these departures from physiological conditions on the effects of modulating the expression of PrP^C is unknown.

It is likely that the roles of PrP^C on the sensitivity to cell death may differ among cell lines, between either isolated cells or complex tissues, as well as depending on the availability of PrP^C ligands. In fact, contradictory functions of cell surface molecules involved in neurodegeneration are not limited to PrP^C. For example, both excitotoxic and neuroprotective roles have been demonstrated for NMDA-type glutamate receptors (see Refs. 295, 476 for reviews), at least in part depending on neurotransmitter concentration (448). Moreover, the role of the low-affinity NGF receptor $p75^{NGFR}$, whether neuroprotective or proapoptotic, depends on its association with distinct partners, most notably the high-affinity TrkA neurotrophin receptor (see Refs. 84, 391 for reviews).

Indeed, both a PrP^C-binding peptide and the PrP^C-binding hop/STI1 cochaperone protected undifferentiated postmitotic cells in retinal explants, as well as dissociated hippocampal neurons subject to induced cell death. Neuroprotection depended on the binding of specific domains in both proteins: $STI1_{230–245}$ and $PrP^C{}_{113–128}$ (92, 306, 591). Other studies also suggest that interaction of hop/STI1 with PrP^C induces cytoprotective signals in a PrP^C-transfected neuronal cell line, through regulation of SOD activity involving the NH_2-terminal domain of PrP^C (463–465).

In vivo cross-linking of PrP^C with two monoclonal antibodies targeting PrP^C epitopes within the range of aa 95–105 induced cell death within the hippocampus and cerebellum of WT mice, whereas both their monovalent Fab fragments and an antibody targeting aa 133–157 were innocuous (497). The authors suggested that dimerization of PrP^C initiates an apoptotic cascade, possibly through an as yet unidentified secondary molecule. Interruption of PrP^C-mediated survival signaling produced by association of PrP^C with another molecule was dismissed based on

the lack of an effect of the Fab fragments. The latter argument, however, is far from compelling, since the breaking of sustained ligand-induced signaling cannot be excluded amidst the widespread effects of antibody cross-linking, particularly the extensive lateral reorganization of cell surface molecules (e.g., Ref. 512; see sect. v*B*).

Sensitivity to cell death is critically dependent on cell-cell interactions, and the mechanisms of cell death or survival of individual cells in a complex, richly interconnecting tissue such as the brain are strongly dependent on the integrity of the tissue (295, 296, 440). Thereby, studies of isolated cells, such as cell lines or cultures of dissociated tissues, must be viewed with caution, because of the intrinsic rupture of histotypical modulators of cell metabolism (208, 297, 372).

A final conclusion about the roles of PrP^C expression, soluble PrP^C and PrP^C ligands on cell death requires further analysis in preparations more akin to their physiological context. Still, the available data indicate that PrP^C is a critical element of the network that controls the sensitivity to programmed cell death in both the nervous system and among other cell types. The outcome of the engagement of PrP^C on either cell death or survival is likely dictated by its available ligands, which in turn determine the array of intervening signaling pathways.

VIII. SIGNALING MEDIATED BY THE PRION PROTEIN

A. Approaches to PrP^C-Mediated Signal Transduction

Activation of certain signal transduction pathways by engagement of PrP^C was demonstrated through direct measurements. In other cases, evidence was inferred from the effect of pharmacological or molecular inhibitors on cellular responses that depend on PrP^C. The latter approach is less reliable, because it may, in fact, disclose signaling pathways networked to the downstream responses, but not directly activated by engagement of PrP^C. This is particularly critical in the case of neurotrophic responses, given the evidence that cell death may be triggered by single events superimposed on a metabolic state controlled by multiple mechanisms (99).

Engagement of PrP^C with either ligands or antibodies, as well as exposure of cells to recombinant PrP^C, have been used in various experimental designs (Fig. 8). The results must be interpreted with caution, due to the singularities of either cross-linking or non-cross-linking ligands (e.g., Ref. 19) and the possibility of either blocking or agonist effects of antibodies (e.g., Ref. 496). Other studies compared signaling intermediates between WT

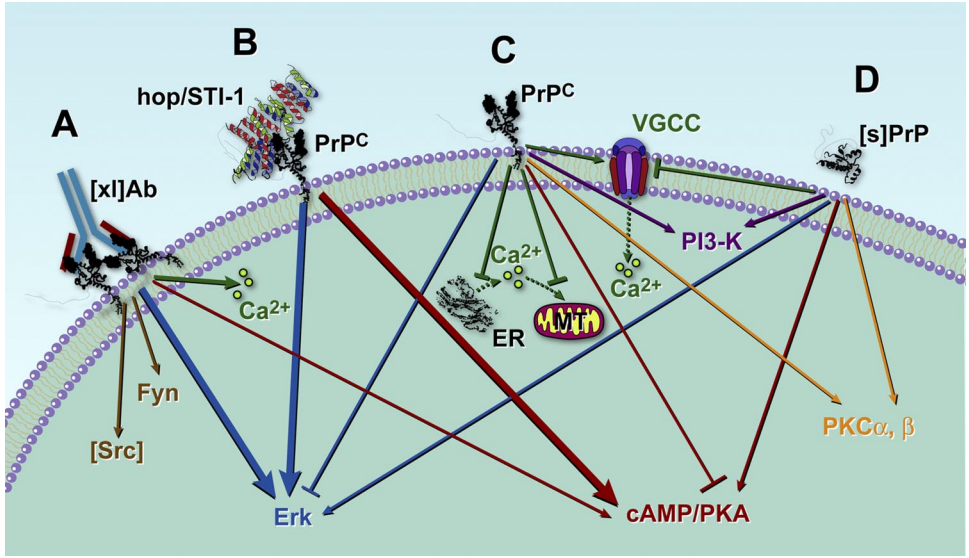

FIG. 8. PrP^C-mediated signaling. The diagram summarizes evidence for modulation of signal transduction by either the expression or the engagement of the prion protein, obtained from a variety of preparations. *A–D* indicate the four major procedures employed in the experimental studies. *A*: cross-linking of PrP^C with antibodies. *B*: engagement of PrP^C with a ligand (hop/STI1). *C*: modulation of the expression of PrP^C or comparison of wild-type and PrP-null mice. *D*: cellular engagement with soluble PrP^C. Arrows indicate activation, hammers indicate inhibition, and each pathway is depicted in a distinct color. The thickness of the lines is an indication of the strength of the evidence, considering the highest when direct evidence of changes in the activity of the pathway was demonstrated. Interrupted arrows indicate calcium fluxes. [xl]Ab, cross-linking antibody; [Src], unidentified Src family tyrosine kinase, distinct from Fyn; ER, endoplasmic reticulum; MITO, mitochondria; VGCC, voltage-gated calcium channel; [s]PrP, soluble PrP, either as recombinant protein or as recombinant fusion PrP-Fc protein. [Drawing of GPI-anchored, glycosylated PrP^C from Jackson and Clarke (235); soluble PrP from Zahn et al. (590); hop/STI1 is a rough sketch composed of the backbones of the three TPR domains (478).] The drawings of the molecules are merely illustrative and are not intended to represent actual models, nor relative sizes.

and PrP-null cells or mice, which may be affected by compensatory responses to deletion of PrP^C. Although the work provided evidence that PrP^C relates to a variety of signaling pathways, fundamental questions remain unanswered as to how the GPI-anchored PrP^C may transfer signals across the plasma membrane.

Studies of the PrP^C peptide 106–126 are not considered here, because most data likely resulted from toxic effects of aggregated peptide, rather than exposure to a specific domain of normal PrP^C (140). Indeed, freshly dissolved $PrP^C_{106-126}$ had neither a toxic nor a protective effect on retinal tissue in vitro (92).

B. Signaling Dependent on PrP^C-Caveolin Interaction

A cell line (1C11) derived from transformation of F9 embryonic carcinoma cells with SV40 T antigen, which can be induced to differentiate along either serotonergic or catecholaminergic phenotypes, was used to test for PrP^C-mediated signaling. Cross-linking of PrP^C induced activation of the Fyn non-receptor tyrosine kinase, which was abolished by antibodies to caveolin-1, and reduced by antibodies to clathrin. The effect was restricted to neurites and growth cones of neurochemically differentiated cells (374).

This suggested that caveolin mediates signaling triggered by engagement of PrP^C in cells that express both proteins. However, the functional significance of this interaction is likely not universal. Caveolin has been demonstrated in neurons after a few weeks in culture (62, 165) and in brain extracts (165, 536), and deletion of the *cav-1* gene produces neurological deficits in mice (536). However, it is still not known whether mature neurons in situ express caveolin (277). It was suggested that, in the absence of caveolin, interaction of PrP^C with gangliosides might be responsible for transferring signals across the membrane (343).

Moreover, caveolins are predicted to attach to the plasma membrane through a hydrophobic hairpin, without traversing the bilayer to the extracellular side (101, 579) and, therefore, can interact directly only with the GPI anchor, not with the polypeptide chain of PrP^C. Caveolins bind cholesterol strongly (379), which is required for membrane incorporation of the protein (290), and transport cholesterol from the ER to membrane caveolae (101), but caveolin has not been shown to bind the stearic acid chains of the GPI anchor of PrP^C (502).

Caveolin-mediated signaling induced by PrP^C cross-linking may, thus, require an intermediate component. NCAM, engagement of which induces phosphorylation of Fyn, is a candidate PrP^C-binding transmembrane, signaling protein (471; see sect. VIII*E*). Nonetheless, biochemical and coimmunoprecipitation procedures indicated that DRMs from growth cones are heterogeneous with regard to their content of distinct NCAM species, and that caveolin segregates from phosphorylated Fyn (196).

Overall, current data fail to support a simple model of signaling from cross-linked PrP^c to Fyn through caveolin. An alternative mechanism is that cross-linking of PrP^C induces a rearrangement of membrane rafts through lateral movement of distinct domains (345, 524), which may involve caveolin in some cells, and then, probably through a transmembrane protein such as NCAM, may couple the GPI-anchored prion protein with the acylated non-receptor tyrosine kinase on the cytoplasmic leaflet of the bilayer.

C. PrP^C, cAMP, and Protein Kinase A

Direct evidence that PrP^C mediates activation of the cAMP/protein kinase A (PKA) pathway was obtained with a peptide that both binds PrP^C (338) and induces neuroprotection in retinal tissue (92). Exposure of developing retinal tissue from WT, but not from PrP-null mice, to the PrP^C-binding peptide increased intracellular cAMP and led to activation of PKA. Neuroprotection induced by both the PrP^C-binding peptide, as well as by an analogous peptide corresponding to the PrP^C-binding domain of protein hop/STI1 (591), was abrogated by blockade of the PKA pathway (Ref. 92 and unpublished data). Protection by hop/STI1 of hippocampal neurons in culture was also blocked by a PKA inhibitor (306). Interestingly, the basal levels of both intracellular cAMP and PKA activity are higher in PrP-null retinal tissue than in WT (92), which likely represents a compensatory response to the lack of PrP^C.

A PKA inhibitor also blocked both neurite outgrowth and neuronal survival of cerebellar granule cells, induced by *trans*-interacting PrP^C (86). However, it was not reported whether the *trans*-interacting PrP^C leads to increased cAMP. In contrast, a PKA inhibitor had no effect on axon outgrowth induced by recombinant PrP^C upon embryonic hippocampal neurons in culture (249).

It is unclear how the engagement of PrP^C or its presence in the extracellular environment leads to increased cAMP. Adenylyl cyclase is commonly targeted by heterotrimeric G proteins engaged by GPCR (248, 310). Both GPCR and G proteins might serve as intermediates in PrP^C-mediated production of cAMP. Indeed, heterotrimeric G proteins reportedly associate with certain GPI-anchored proteins in membrane rafts (300, 397, 498, 499), but no such evidence is available for PrP^C. The identification of a GASP as a PrP^C ligand (see sect. II*D*) may be of significance, but the topologies of PrP^C and GASP are not consistent with direct interaction.

In the 1C11 cell line differentiated along the serotonergic phenotype, ligation of PrP^C with antibodies par-

tially blocked the reduction in cAMP content induced by agonist binding to 5-$HT_{1B/D}$ receptors, an effect attributed to interference with a complex cross-talk among various 5-HT receptor types (376). This indicates that PrP^C can modulate the production of cAMP triggered by engagement of GPCR. Still, it is not known whether PrP^C and the 5-HT receptors cohabit the same membrane compartments, nor whether they undergo physical interaction. In this study, as in many others, the PrP^C antibodies induce clustering of the protein (365), which may underlie the observed effects, but raises some concern as to physiological relevance.

Notwithstanding, distinct members of the adenylyl cyclase family include not only transmembrane, but also soluble forms, and both classes are regulated by additional factors, such as calcium and calmodulin (113, 248). Calcium-stimulated isoforms were identified in both adult and developing rodent retina (1, 441), and both the mRNA for a calcium-stimulated adenylyl cyclase and the calcium-induced accumulation of cAMP were detected, albeit at low levels, in the cerebellum (342), at a time compatible with the reported cAMP/PKA-dependent responses of cerebellar granule cells induced by *trans*-interacting PrP^C (86). Thus it cannot be ruled out that PrP^C-dependent activation of the cAMP/PKA pathway is mediated by either voltage-gated or store-operated (capacitative) calcium channels at the plasma membrane (113). Finally, a direct interaction of PrP^C with transmembrane forms of adenylyl cyclase also cannot be excluded.

In summary, although PrP^C clearly modulates the cAMP/PKA pathway with significant biological consequences, the mechanism of signal transfer across the plasma membrane remains unknown. An additional caveat must be raised, in that biochemical measurements of cAMP, as well as pharmacological inhibitors of PKA, override subcellular compartmentalization, a particularly relevant issue due to the importance of both PrP^C localization and trafficking, as well as of differential localization of adenylyl cyclases for their physiological functions (113, 248, 522).

D. PrP^C and MAP Kinases

Treatment of developing retinal tissue with PrP^C-binding peptides led to activation of Erk (92), and the same occurred in cultured hippocampal neurons treated with either hop/STI1 or with the PrP^C-binding peptide hop/$STI1_{230–245}$ (306). In the latter cells, neuronal differentiation induced by hop/STI1 was abrogated by an Erk inhibitor, differing from axon outgrowth induced by recombinant PrP^C upon embryonic rat hippocampal neurons, in which Erk inhibition had no effect (249). No Erk activation was detected in PrP-null retina or hippocampal neurons treated with hop/STI1. Interestingly, basal Erk phosphorylation was higher in PrP-null retinae and hippocampal neurons than in WT (92, 306), a result that was also found in extracts from either total brain or cultured cerebellar cells (51) and is apparent in studies of splenocytes from either genotype (348). This increased basal activity of Erk may also represent a compensatory response to the deletion of *Prnp*.

Antibody-induced clustering of PrP^C led to phosphorylation of Erk1/2, but neither of Jun NH_2-terminal kinase nor of the stress-activated p38 kinase, in the 1C11 cell line and both its differentiated derivatives. Similar Erk activation was found in the mouse neurohypothalamic GTI-7 cell line, the lymphoid BW5147 cell line, and the Jurkat T cell line but not in GPI-deficient cells, primary embryonic cortical or hippocampal neuron cultures, and rat neuroblastoma B104 cells (365, 480, 512). Erk phosphorylation was prevented by blocking the activity of NADPH oxidase, which also follows PrP^C cross-linking, thus indicating that the production of ROS may be an intermediate step in the PrP^C-dependent activation of Erk (480). In the same study, phorbol ester also led to activation of the NADPH oxidase-Erk cascade, although the interpretation that activation of protein kinase C by engagement of PrP^C is an intermediate step in this response is not warranted (254). In turn, both ROS production and Erk phosphorylation in 1C11-derived differentiated cells were prevented by PP2, consistent with differentiation-dependent intermediation of Fyn kinase (480).

The fact that pharmacological inhibition of NADPH oxidase did not completely block Erk phosphorylation in the differentiated 1C11 derivatives was taken as evidence for an additional NADPH oxidase-independent pathway (480). Indeed, a specific antagonist of the epidermal growth factor (EGF) receptor blocked Erk phosphorylation induced by PrP^C cross-linking in the GTI-1 cell line, suggesting that PrP^C clustering leads to transactivation of the EGF receptor, which might then lead to Erk phosphorylation (365). However, NADPH oxidase inhibition completely abolished Erk phosphorylation in the same cell line (480), and EGF receptor ligation also leads to the production of ROS (531).

On the other hand, a PrP^C-Fc fusion protein was shown to induce phosphorylation of Erk1/2 in the macrophage-like P388D-1 cell line, which was reduced by either the Src-family kinase inhibitor protein phosphatase 1 or by the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin (268). Although the use of a control Fc domain allowed the extraction of PrP^C-derived responses, it was not clear whether Fc receptors in the macrophage-like cells may have interfered with PrP^C binding to cell surface partners.

Thus both engagement of PrP^C at the cell surface as well as exposure to extracellular PrP^C induce Erk activation, and expression of PrP^C affects the basal level of Erk activity. A canonical pathway of Erk activation includes

the activation of the Ras GTPases, following binding of the SH2-containing Grb2 adaptor protein to phosphorylated cytoplasmic domains of receptor tyrosine kinases. PrP^C bound Grb in a two-hybrid screen (500), but the topology of both proteins seems incompatible with a functional role for this binding. Adding to the canonical pathway, an active cAMP/PKA pathway may either activate or inhibit the Erk pathway, changes in intracellular calcium (see sect. VIII*F*) can indirectly modulate Erk activity, and protein kinase C (see sect. VIII*G*) can lead to Erk activation by either a Ras-dependent mechanism or directly through activation of Raf-1 (see Refs. 186, 310, 510 for reviews). Therefore, the activation of Erk1/2 may be secondary to various upstream events. Still, the question of how PrP^C interacts with any or all of these upstream intracellular signaling components remains unsolved.

E. PrP^C and Non-Receptor Tyrosine Kinases

As mentioned above, antibody cross-linking of PrP^C in differentiated 1C11 cells led to phosphorylation of Fyn (374), and the Fyn kinase inhibitor PP2 abrogated downstream phosphorylation of Erk (480). Other studies provided evidence that cross-linking of PrP^C recruits non-receptor tyrosine kinases, such as Fyn and Lck, to PrP^C-containing membrane rafts (512), but their state of activation was not reported.

Axon outgrowth induced by recombinant PrP^C upon embryonic rat hippocampal neurons in culture was impaired by both the general Src kinase family inhibitor herbimycin, as well as by the Fyn inhibitor PP2 (249). In contrast, PrP^C cross-linking in CEM-T lymphoid cells produced phosphorylation of a Src-family kinase distinct from Fyn (225). Biochemical studies of cerebellar granule cells also failed to confirm an association of PrP^C with Fyn (38). In addition, exposure of a macrophage cell line to PrP^C-Fc induced phosphorylation of the non-receptor tyrosine kinases Syk and Pyk2, as well as the adaptor protein Cbl, but Fyn was not affected (268).

Thus it appears that recruitment and activation of non-receptor tyrosine kinases by PrP^C may be a component of PrP^C-mediated signaling, but the recruited and/or activated kinases appear to vary either among distinct cell types or depending on particular conditions.

As for the signal transfer from PrP^C to Fyn, functional studies provided strong evidence that PrP^C is able to recruit and stabilize NCAMs into lipid rafts upon stimulation of NCAM, which appears to be required for the ensuing activation of Fyn by the complex formed by NCAM and the receptor type protein phosphatase α (471). Nonetheless, since PrP^C and Fyn appear to reside in distinct membrane domains (38), this interpretation requires either fusion of membrane domains or lateral trafficking of protein components.

F. PrP^C and Calcium

Reduced calcium influx through VGCC was found both in cultured cerebellar granule cells and in hippocampal neurons in brain slices of PrP-null, compared with WT mice (161, 199), and exposure of WT cerebellar granule cells to PrP^C reduced VGCC (266). Cells transfected with *Prnp* presented an increased agonist-induced calcium influx through the plasma membrane, while reducing the release of Ca^{2+} from the ER and Ca^{2+} uptake by mitochondria (44, 259). In contrast, ConA-induced calcium fluxes were similar in both WT and PrP-null splenocytes (348).

Engagement of PrP^C by specific antibodies increased cytosolic calcium in CEM-T cells (225). More importantly, cross-linking of PrP^C in Jurkat cells induced a transient Ca^{2+} signal, which is itself necessary for the ensuing PrP^C capping events, because coincubation with the Ca^{2+} chelator BAPTA-AM prevented the recruitment of PrP^C and other proteins into the caps (512; see sect. V*B*). These results show that an early Ca^{2+} signal induced by PrP^c cross-linking feeds back into further events dependent on lateral redistribution of cell surface proteins, including the lateral trafficking of PrP^C itself.

Thus PrP^C is associated with calcium-mediated cellular events, and calcium channels may be transmembrane partners of PrP^C-mediated signaling. However, no evidence is available to date of direct physical interaction of PrP^C with calcium channels at the plasma membrane.

G. PrP^C and Protein Kinase C

Protein kinase C (PKC) comprises various subfamilies, either calcium dependent or independent (125). A few studies provided evidence that PrP^C may lead to activation of PKC. Thus both proteins were recovered from the same fractions of immunoprecipitated DRMs (38), and axon outgrowth induced by soluble PrP^C in embryonic rat hippocampal neurons in vitro was blocked by a wide-spectrum PKC inhibitor (249).

In splenocytes, the level of lectin-induced phosphorylation of the calcium-dependent PKC-α and PKC-β forms, but not the calcium-independent PKC-δ form, were reduced in PrP-null cells, compared with WT. This was not observed in response to phorbol ester, nor were there differences between lectin-induced calcium fluxes in PrP-null and WT splenocytes. These data indicate that the presence of PrP^C affects signaling related to phosphorylation of some forms of PKC, which may be related to their function in the transduction of signals initiated at the level of the plasma membrane (348). The link between PrP^C and PKC remains, however, undetermined.

H. PrP^C and the PI 3-kinase/Akt Pathway

The activity of PI 3-kinase (PI3-K) was higher in the brains of WT than PrP-null mice, and in neural cell lines transfected with *Prnp* compared with parental cells. In *Prnp*-transfected cell lines, the activity of PI3-K as well as cytoprotection conferred by the latter against oxidative stress were abrogated by either copper chelation or deletion of the PrP^C NH_2-terminal octarepeat domain, suggesting that this copper-binding domain plays a major role in the effect of PrP^C upon PI3-K (543). In other studies, phosphorylation of Akt was reduced in the brains of PrP-null mice, compared with WT, both in control conditions and early after an ischemic insult (562).

Conversely, PI3-K inhibitors partially blocked the axon outgrowth of hippocampal neurons induced by recombinant PrP^C (249), as well as neuronal survival, but not neurite outgrowth induced by PrP^C-Fc upon cerebellar granule cells (86). In the macrophage-like P388D-1 cell line, this same PrP^C-Fc fusion protein induced a slightly higher level of Akt phosphorylation than control Fc fragment, and Akt phosphorylation was completely blocked by Src-kinase inhibitor protein phosphatase 1 (268). Interestingly, in the latter study, both a PI3-K inhibitor diminished Erk phosphorylation as well as an Erk inhibitor inhibited Akt phosphorylation induced by the fusion protein, suggesting reciprocal interactions of the two pathways upon PrP^C-mediated signaling.

I. Implications of Current Evidence on PrP^C-Mediated Signaling

Modern approaches to cell signaling emphasize networks, rather than independent pathways (122, 238, 431). However, studies of PrP^C-mediated signaling have usually been directed at individual responses (Fig. 8). When several signals were induced, little effort was made to examine their interdependency, and in certain cases, the sequence of activation of signaling cascades was assumed, but not adequately proven.

Further analysis of signaling mediated by the prion protein is likely to unravel the mechanisms by which modulation of expression, engagement, or exposure to soluble PrP^C trigger proliferative, differentiating, or death/survival responses, as well as other effects on cell metabolism, such as modulation of responses to oxidative stress, synaptic modulation, and immunomodulation. These, in turn, will likely explain the systems-level functions of PrP^C. An integrated systems approach may help defining signaling patterns generated by multiple pathways.

Nevertheless, the major conundrum of signal transfer from PrP^C to intracellular compartments still remains unsolved and may benefit from novel approaches. In particular, although the need for transmembrane signaling partners of PrP^C has long been recognized, the evidence that PrP^C binds a variety of ligands, several of which may fulfill the requirement for a signaling intermediate and others may bridge PrP^C with further transmembrane partners (see below), likely requires the abandonment of the idea that a single intermediate molecule may explain the multiple PrP^C-mediated signals already demonstrated in many distinct models.

IX. THE PRION PROTEIN AS A DYNAMIC CELL SURFACE PLATFORM FOR THE ASSEMBLY OF SIGNALING MODULES

The extensive, and often contradictory, data reviewed above appear to draw a disappointingly dispersive picture of the functions of this protein. It may be questioned whether PrP^C can be ascribed a specific function, or, instead, inserts as a wild card in a variety of functional processes. Indeed, some of the data obtained at the cellular level suggest that, rather than playing a specific role in a straightforward signaling pathway, PrP^C may serve as a scaffolding protein in multiple sets of as yet poorly defined interactors at the cell surface. Moreover, both the endogenous, as well as the ligand-induced, trafficking of PrP^C may strongly affect its functions according to the immediate membrane environment.

Some basic features of PrP^C favor such a dynamic scaffolding of multicomponent complexes. Distal to the lipid bilayer, specific PrP^C domains bind various proteins, glycosaminoglycans, and metal ions (see sect. II*D*, Fig. 3). Current data preclude the accreditation of a major, let alone a single relevant ligand. Since the binding domains of PrP^C are distinct for at least some of these ligands, and the affinity constants for confirmed ligands are variable, each cell likely interacts with a distinct set of partners through PrP^C, depending on both the level of expression of the latter and the characteristics of the immediate microenvironment (Fig. 9). The evidence for potential involvement of PrP^C in multiple signaling pathways (sect. VIII, Fig. 8) also cautions against the interpretation that a single PrP^C ligand may explain complex phenotypes associated with various manipulations of either the expression or the structure of PrP^C (21, 287, 484).

Proximally, the GPI anchor and ensuing raft association, coupled with both the lateral and endocytic trafficking of PrP^C, also favor a dynamic scaffolding function (Fig. 9). Whether located within or outside lipid rafts, PrP^C is due to encounter distinct membrane or cell surface proteins (154, 273, 385, 481, 549), some of which may momentarily bind the trafficking PrP^C. Conversely, cross-linking or otherwise ligand-induced dragging of PrP^C is likely to affect its kinetics of lateral trafficking and asso-

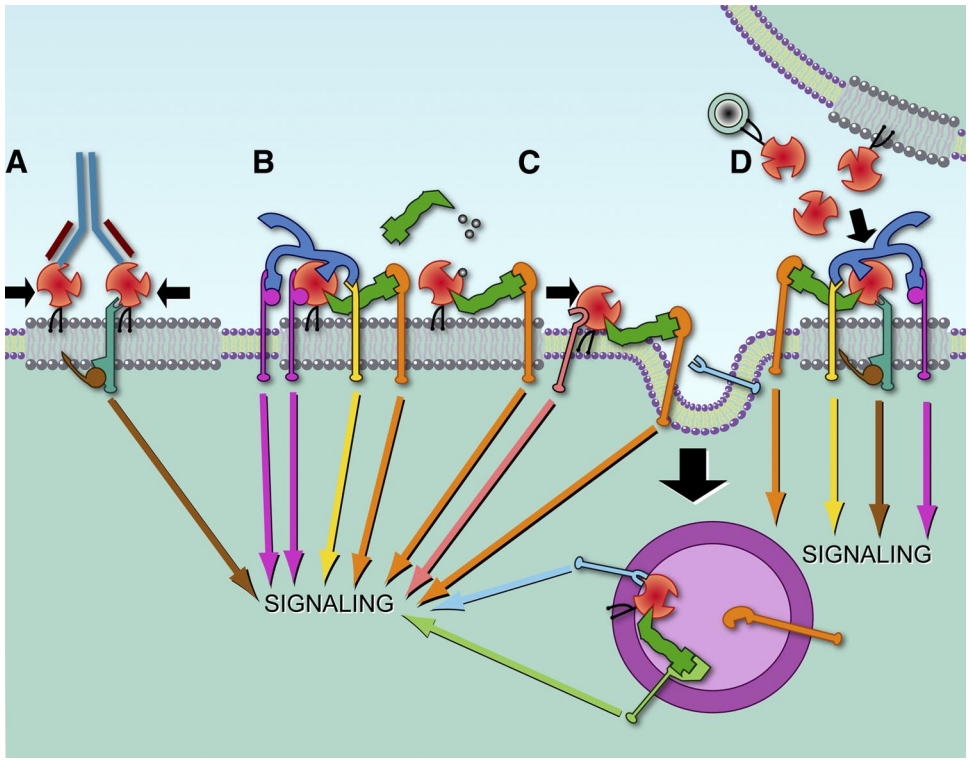

FIG. 9. Model of PrP^C as a cell surface platform for the assembly of signaling modules. The plasma membrane is represented by the lipid bilayer, an intracellular endocytic vesicle by continuous lines, and lipid rafts by a gray-shaded thicker bilayer. The cytoplasm is colored light green. PrP^C is shown in red, either GPI-anchored (black line forks) or as a soluble polypeptide. Black arrows indicate either molecular trafficking, at the cell surface and extracellular medium, or endocytic trafficking, intracellularly. *Trans*-acting PrP^C may originate physiologically from a neighboring cell, or from exosomes (*top right*). The dark blue and dark green profiles depict extracellular PrP^C-binding macromolecules. Metals are shown by small circles and, together with macromolecular ligands, can modulate trafficking of PrP^C. Various long, slender shapes and colors represent distinct transmembrane proteins, and the matching colored arrows depict their respective signals. Multiple ligands fit several binding pockets in PrP^C, and bind transmembrane proteins, which leads to signaling either directly or indirectly through signaling cascades (see Fig. 8). *A*: cross-linking approximates PrP^C molecules and may induce raft coalescence, thus approximating binding partners from distinct raft domains. *B*: various combinations of direct PrP^C-binding proteins, or secondary binding proteins, lead to distinct arrays of signals arising from differing plasma membrane domains. *C*: lateral movement of PrP^C changes the array of available ligands, and endocytosis may further switch the ligand profile, at least in part as a function of changing local pH. *D*: *trans*-acting PrP^C functions as a scaffolding protein, similar to GPI-anchored PrP^C, except for the lack both of direct effects upon the lipid bilayer and of lateral trafficking of its own, but it can be dragged by laterally trafficking, transmembrane binding proteins.

ciation. This was shown in other scenarios by the antibody-induced transient anchorage of the GPI-anchored proteins Thy-1 and CD73 (88). Cross-linking is also likely to produce raft coalescence (345, 449). Moreover, engagement of certain membrane receptors produces extensive reorganization of lipid rafts (383), which would affect the probability of raft-associated molecules such as PrP^C to encounter its ligands.

In addition, the fast trafficking of PrP^C between the cell surface and endosomal compartments (sect. III*C*; Fig. 4), as well as the effect of local charged residues at the cell surface (90), impart a continuous variation of local pH, which may also affect the array of complexed PrP^C ligands (Fig. 9), as shown for other ligand-receptor pairs (319). The arrays of ligands selectively available within endosomal compartments may have critical consequences for PrP^C-mediated functions, as shown for other signaling pathways (218, 219). This may explain, for example, the dependence on endocytosis shown for PrP^C-mediated cell death (516), as well as for activation of the Erk pathway by the PrP^C ligand hop/STI1 (8).

Therefore, the ligand binding-dependent movement of PrP^C along a changing microenvironment (sect. III, *C* and *D*), continuously feeds back into the composition of the protein complexes assembled at differing points along the protein's trafficking trajectory. It follows that PrP^C partners, and thus PrP^C-mediated signaling, may change significantly along trafficking pathways. Interestingly, dependency on both lateral and endocytic trafficking on PrP^C function may even explain certain discrepancies reported at the systems level, such as that between LTP in brain slices studied at either physiological or room temperature (108, 302, 332, 577), due to the effect a difference of ~15°C may have on the rate of protein trafficking and raft organization (229, 433, 594).

These properties confer a high degree of complexity to the functions of PrP^C at the cellular and molecular level (Fig. 9). To date, little data are available on the interaction of confirmed PrP^C ligands, but further elements are superimposed by higher order interactors, such as laminin and the LRP, both of which bind PrP^C (171, 183) and each other (79), or laminin-binding integrins, which extend the range of influence of PrP^C. Notably, the binding site in LRP for both laminin and PrP^C is the same (443), and the binding sites in PrP^C for both LRP and laminin partially overlap (103, 183, 226; see Fig. 3), which implies an intricate kinetics of binding among the three proteins at the cell surface, as well as challenging structural requirements.

Engagement of GPI-anchored PrP^C, as well as treatment of cells with soluble PrP^C, often activate similar signaling pathways (sect. VIII) and produce similar biological effects, such as cell differentiation or survival (sect. VII). Although it is not clear whether soluble PrP^C occurs in physiological conditions, both direct contacts between PrP^C-expressing cells as well as secretion of PrP^C associated with exosomes (see sect. III*B*) may represent a physiological counterpart of the soluble PrP^C used in experiments. This is also consistent with the idea that the polypeptide chain of PrP^C may scaffold multicomponent complexes. Thus both cross-linking agents, such as lectins or antibodies, as well as PrP^C added to the extracellular medium, increase the concentration of PrP^C locally at the cell surface, with consequences for the binding of ligands with distinct affinity constants. The latter is also a function of the level of expression of PrP^C, which may vary for individual cells depending on cell differentiation or environmental conditions. If, indeed, PrP^C partners may vary qualitatively as a function of expression of PrP^C, then data on various transgenic animals, as well as on distinct preparations of transfected cells, should be viewed with additional caution.

Dragging of PrP^C by an extracellular matrix molecule such as laminin should bring PrP^C closer to another ligand, the laminin receptor, and similar effects would be expected from other PrP^C ligands, such as hop/STI1, with regard to the latter's own additional ligands. The case of hop/STI1 is somewhat similar to the soluble PrP^C, in that it may serve as a bridge between transmembrane and/or cell surface molecules, including GPI-anchored PrP^C itself. Importantly, the arrays of putative ligands identified to date for PrP^C (sect. II*D*), as well as for its direct ligands, such as hop/STI1 (394), were derived from experimental approaches aimed at each individual protein. It is possible that binding of PrP^C to its ligands may allow the co-option of novel higher-order ligands into the complex, for example, as a consequence of changes in the structure of each or both binding partners (e.g., Ref. 11). Indeed, biophysical methods show that the structure of PrP^C is affected upon binding of its ligand hop/STI1 (S. A. Romano, Y. Cordeiro, L. M. Trambaioli, D. Foguel, and R. Linden, unpublished results).

These considerations suggest that PrP^C may, indeed, function at the cell surface as a dynamic platform for the assembly of various signaling modules (Fig. 9). This may explain why so many individual signal transduction pathways have already been shown to underlie PrP^C-mediated effects (sect. VIII), as well as the multiplicity of seemingly unrelated functions in various cell types.

X. CONCLUSION AND FUTURE DIRECTIONS

Fifteen years have elapsed since the first PrP-null mouse (63) offered a decisive contribution to the understanding of prion pathology. The lag between the ensuing gain-of-function hypothesis of prion diseases and the question of PrP^C physiological functions has only in part been compensated by recent efforts in many laboratories around the world.

Notwithstanding, there is now overwhelming evidence that PrP^C is involved in a variety of important physiological properties at the systemic, cellular, and molecular levels, within the nervous and immune systems, and probably in other organs. A particularly recurrent theme among these properties appears to be the processing of either systemic or cellular stress and danger signals, but the growing literature on both transgenic and pharmacological/biochemical approaches to PrP^C suggests that many additional putative systemic functions are likely to emerge in the near future.

In turn, the physiological approach to PrP^C bears upon the fundamental understanding of membrane biology and signal transduction. The current review suggests that a fresh perspective on functions of PrP^C depends on further studies of *1*) the properties of PrP^C ligands, including the latter's' own ligand profiles; *2*) the structural basis of PrP^C-ligand interactions and their consequences; *3*) the regulation of the expression of PrP^C in physiological context; *4*) the effects of PrP^C ligands on the trafficking of PrP^C; and *5*) the consequences of molecular trafficking on PrP^C-based multicomponent complexes and signal transduction.

Experiments along these lines should provide a clearer picture as to why PrP^C differentially affects such varied functional properties at the systems level, how PrP^C-mediated cellular responses explain systemic effects, which and how signaling pathways underlie responses at the cellular level, and the molecular and structural interactions associated with PrP^C functions. The latter are particularly important for the eventual modulation of physiological functions of PrP^C.

The generalized view of PrP^C as a dynamic platform for the assembly of signaling modules at the cell surface

(Fig. 9) extends the notion of scaffolding proteins involved in the assembly of intracellular pathways of signal transduction (43, 168, 265, 435, 483, 522). On the basis of this concept, further studies of PrP^C-mediated molecular interactions and transmembrane signaling should help clarify the most important questions within the framework of physiological functions of the prion protein. Explaining these properties in the light of the loss-of-function hypothesis may have a decisive impact on the design of novel therapeutic approaches for prion diseases.

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GRANTS

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