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Distribution and Function of the Normal Cellular Isoform of the Prion Protein

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Table of Contents

Index of Figure Legends

Acknowledgments

This is essentially the most personal and the most important part of this thesis, where recognition is given to those who made it all possible. Without these very special folks, the last 6 years would not have been the same.

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Moi qui souris
Qui marche enfin
La tête hors des épaules
Libre dans un corps
Moi qui jaillis
Des souterrains
De cent siècles de taule
Libre à mourir de rire

Daniel Bélanger

Ouf, c'est fini!

Preface

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, an a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Abstract

The normal isoform of the prion protein (PrP^C) is a small cell-surface glycoprotein that is expressed predominantly in the brain as well as a wide variety of peripheral tissues. Although the involvement of an abnormal, protease-resistant isoform of the protein (PrP^{Sc}) in certain neurodegenerative diseases is clear, its normal function remains unknown. We investigated several aspects of prion protein biology to improve our understanding of the role it may play in both health and disease.

We characterized the expression pattern of PrP^C during human leukocyte maturation. We observed that prion protein is present on bone marrow stem cells and that it is downregulated upon differentiation along the granulocyte lineage *in vivo*. This observation was replicated using an *in vitro* system of granulocyte differentiation. These data suggest that bone marrow stem cells may support prion agent replication in disease, since this process is dependent on availability of PrP^C. Additionally, our studies provide a novel *in vitro* system to study prion gene regulation.

We developed soluble, tagged versions of the prion protein to determine the existence of a cognate cell-surface receptor. A prion-human placental alkaline phosphatase fusion protein was found to bind specifically to the surface of many cell lines, implicating the existence of such a receptor.

We further investigated the nature of this interaction and determined that it was not only specific, but saturable and high-affinity, as expected for a ligand-receptor interaction. We determined that the N-terminal region of the prion protein is essential for binding to the receptor and mapped the precise location of the binding site using peptide inhibition studies. It was found that the N-terminal region will interact with the receptor only when bound by copper ions, suggesting that PrP^C and its receptor may play a role in copper regulation.

We developed a novel expression cloning methodology using Xenopus oocytes to identify the receptor for PrP. Protocadherin 2 was isolated as a positive clone and is therefore proposed as a prion receptor candidate.

Résumé

L'isoforme normale de la protéine prion (PrP^C) est une petite glycoprotéine, présente à la surface cellulaire, exprimée principalement dans le cerveau et dans une grande variété de tissus périphériques. Bien que l'on ait établi clairement le lien entre l'isoforme anormale de la protéine, résistante aux protéases (PrP^{Sc}), et certaines maladies neurodégénératives, la fonction de la protéine normale demeure toutefois inconnue. Nous avons étudié plusieurs aspects de la biologie de la protéine prion afin de mieux connaître le rôle qu'elle pourrait jouer tant dans un organisme sain que dans un organisme malade.

Nous avons caractérisé l'expression de la PrP^C au cours de la maturation de leucocytes humains. Nous avons observé que la protéine prion est présente sur les cellules de souche de la moëlle osseuse et qu'elle est rétrorégulée lors de la différenciation pendant le lignage granulocytique *in vivo*. Cette observation a été reproduite en utilisant un système *in vitro* de différenciation des granulocytes. Ces données suggèrent que les cellules de souche de la moëlle osseuse pourraient entretenir la reproduction de l'agent prion dans l'organisme malade puisque le processus dépend de sa présence. De plus, nos recherches fournissent un systeme *in vitro* original permettant d'étudier la régulation génétique du prion.

Nous avons préparé des versions solubles et marquées du prion pour déterminer l'existence d'un recepteur de surface cellulaire apparenté. Nous avons découvert qu'une protéine de fusion prion-phosphatase alcaline du placenta humain (PrP-AP) se liait spécifiquement à la surface de nombreuses lignées cellulaires. Nous avons ainsi mis en évidence l'existence d'un tel récepteur.

Nous avons étudié par la suite la nature de cette interaction et déterminé qu'elle était non seulement spécifique, mais saturable et de haute affinité ainsi que l'on pouvait s'y attendre dans une interaction de type récepteur/ligand. Nous avons déterminé que la région terminale N de la protéine prion est essentielle pour se lier au récepteur et nous avons établi la location précise du site de liaison en utilisant des études d'inhibition par peptides. Nous avons trouvé que la région terminale N n'interagissait avec le récepteur que lorsqu'elle était liée par des ions cuivre, ce qui suggère que la protéine PrP^{C} et son récepteur pourraient jouer un rôle dans la régulation du cuivre.

Nous avons établi une méthodologie originale de clonage d'expression en utilisant les oocytes Xenopus pour identifier le récepteur de la PrP. Le résultat du tri a permis l'isolation d'un clone de la protocadhérine deux, que nous proposons comme candidat récepteur de la protéine prion.

Abbreviations & Designations

Ab	antibody
BSA	bovine serum albumin
CD15	cell surface carbohydrate marker, aka SSEA-1
CD34	bone marrow stem cell marker
CJD	Creutzfeldt-Jacob disease
CNS	central nervous system
FSC	forward scatter
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
mAb	monoclonal antibody
mw	molecular weight
PC2	protocadherin 2
PIPLC	phosphatidylinositol phospholipase C
PrP	prion protein
PrP^{C}	normal cellular isoform of prion protein
PrP^{Sc}	scrapie isoform of prion protein
PrP	prion construct lacking GPI anchor sequence
PrP-AP	prion-human placental alkaline phosphatase fusion protein
PrP-Ig	prion-immunoglobulin fusion protein
PrP 27-30	protease resistant core of PrPSc
SSC	side scatter
SOD-1	superoxide dismutase
SSEA-1	stage specific embryonic antigen 1
TSE	transmissible spongiform encephalopathies

Claims to Originality

For all the work presented within this thesis, the author is the primary researcher and experimenter. Contribution by co-authors are explicitly stated at the beginning of each chapter concerned (Chapters 4 & 5) The following are the claims to originality contained within:

Prion Protein Expression in Human Leukocyte Differentiation

1) I have shown that, of all mature leukocytes, only granulocytes do not express PrP^C, but that bone marrow stem cells are positive for PrP^C expression, indicating a negative regulation of PrP during differentiation of the granulocyte lineage. This was confirmed by analysis of immediately *ex vivo* differentiating granulocytes. This study expands our knowledge of the expression patterns of PrP^C, and provides important information with regards to the infectious potential of human and animal bone marrow.

2) An *in vitro* differentiation model using the HL-60 cell line was used to demonstrate that expression of PrP^C in the granulocyte lineage is regulated at the transcriptional level. This system provides a model with which to study PrP gene regulation, which has been poorly studied.

Generation of soluble prion protein constructs

3) To determine the existence of a receptor for PrP^C, it was essential to develop specific, easily detectable probes with which to carry out our studies. Both the prion-immunoglobulin (PrP-Ig) and the prion-human placental alkaline phosphatase (PrP-AP) fusion proteins are easily produced, tagged, soluble versions of the prion protein that have not been previously reported in the literature. PrP-AP was used successfully to detect a PrP binding activity on the surface of many cell lines, confirming a unique report in the literature.

Characterization of a cell surface receptor for the prion protein

4) The prion protein binding activity detected using PrP-AP was further characterized. It was determined that this binding activity was specific, saturable and of an affinity ten-fold higher than that stated in the only other report in the literature. The binding activity was also protease sensitive, demonstrating the existence of a prion interacting protein at the cell surface, in other words a receptor. Although postulated in the literature, this is one of only two demonstrations of the existence of such a receptor.

5) It was found that a large panel of cell lines from various tissues exhibited binding of PrP-AP, with muscle cell lines showing the highest activity. The presence of a receptor for prion protein on muscle cells has never been reported.

6) It was determined that the N-terminal region of the prion protein is essential for interaction with the receptor. Peptide inhibition studies precisely mapped the polypeptide necessary for binding. This is the first demonstration of the precise region of the prion protein that is necessary for interaction with its receptor.

7) Although several reports have characterized the binding of copper to the N-terminal octarepeat region of the prion protein, we are the first to show that copper is an essential cofactor for the interaction of prion protein with its receptor.

Cloning of the prion protein receptor

8) We have developed a novel expression cloning methodology using Xenopus oocytes to identify the receptor for the receptor for the prion protein.

9) Having used PrP-AP to screen a muscle cell line expression library, we are the first to report that the protocadherin 2 glycoprotein is a strong candidate for the prion receptor.

Introduction

Although the role of the scrapie isoform of the prion protein, PrP^{Sc}, has been intensely studied in the context of the prion diseases, the biological function of its normal cellular counterpart, PrP^{C}, has proven elusive. Our goal was to investigate aspects of PrP^{C} biology in order to improve our understanding of this highly conserved protein in both health and disease paradigms.

We began our investigations with the examination of PrP^{C} expression patterns during human leukocyte maturation, since there is major concern about transmission of prion disease via the blood supply and the infectious potential of a tissue depends on the presence of PrP^{C}. We determined that, of the mature leukocytes, only granulocytes fail to express PrP^{C}. However, bone marrow stem cells, the precursors of all leukocyte lineages, were positive for PrP^{C}, indicating a negative regulation of this protein during granulocyte maturation. This was found to be the case *in vivo* and was replicated *in vitro*. We propose that the granulocyte model provides a system which which to study the regulation of PrP^{C} expression at a transcriptional level. Additionally, PrP^{C} expression by bone marrow stem cells argues for the PrP^{Sc} infectivity potential of these cells, which should be taken into account for disease prevention (Chapter 2).

The near ubiquitous tissue distribution of PrP^{C} expression, together with its presence on the cell surface, suggests that PrP^{C} functions as

an important mediator of cell adhesion, signal transduction, or uptake of extracellular ligands. We decided to investigate the existence of a receptor for PrP^C, postulating that the identification of such a receptor and the characterization of its interaction with PrP^C would give us clues as to the true function of the protein. The development of sensitive and specific detection tools for this potential receptor was a critical first step. Three different constructs were generated, and a fusion of prion protein and human placental alkaline phosphatase (PrP-AP) proved successful in the detection of a binding activity present on many cell lines (Chapter 3).

Further analyses demonstrated that the binding activity observed is consistent with the existence of a receptor since it is specific, of high affinity and is dependent upon a protein component. Although the presence of the N-terminal region of PrP has been shown to be unnecessary for the disease process, we determined that it is essential for receptor interaction. Since copper has been found to bind to octapeptide repeats within this N-terminal region, we investigated the possible contribution of this metal to PrP-AP/receptor binding. We observed that copper is a necessary co-factor for the activity of this domain and that it may confer a favourable binding conformation (Chapter4). We propose that interaction of PrP with its receptor is a copper dependent mechanism, and may thus serve to regulate the balance of copper *in vivo*.

Several candidate proteins have been proposed in the literature as PrP binding proteins. Although these proteins may interact with PrP in some fashion, none fully satisfy the requirements of a widely expressed, high affinity receptor as revealed by our observations. We therefore carried out a cloning effort to identify the receptor for PrP. Isolation of a clone for protocadherin 2 led us to conclude that this member of a widely expressed family of proteins may be involved with PrP^C interaction (Chapter 5).

Chapter 1

Literature Review

Prion protein in health and disease

The term 'prion' was coined by Dr. Stanley Prusiner to define a novel class of infectious agent, an agent of 'proteinaceous infectious' nature. This agent is the involved in a class of neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs) or prion diseases. The TSEs are a group of human and animal diseases which include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and Fatal Familial Insomnia (FFI) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE), or 'mad cow disease', in cattle. The neuropathology of TSEs is characterized by neuronal loss, spongiform change, astrocytic hypertrophy (gliosis), and accumulation of an abnormal protease-resistant protein designated PrP^{Sc} (for prion protein scrapie) in both brain and spleen (reviewed in (Prusiner, 1998)).

Transmission of disease in laboratory animals using purified preparations of this protease resistant protein led to the proposal that it was the major, if not the only, component of the infectious agent (reviewed in (Prusiner, 1989)). Oligonucleotide probes derived from amino acid sequences of the N-terminus of the purified protein were used to screen a cDNA library from scrapie-infected hamster brain (Oesch et al., 1985; Prusiner et al., 1984). Analysis using

positive cDNA clones revealed that these sequences were present as an identical single copy gene in both normal and scrapie-infected hamsters (Oesch et al., 1985). The cDNA sequence predicted a small protein of just over 250 amino acids with a glycine/proline rich octarepeat region in the N-terminal half and two Asn-linked glycosylation sites and an intracellular disulfide bond in the C-terminal half (Kretzschmar et al., 1986; Turk et al., 1988). The protein itself has been found to be a widely expressed glycoprotein of 33-35 kDa that is anchored to the outer plasma membrane by a glycosylphosphatidyl-inositol anchor. How then could this protein, encoded by a gene present in both normal and diseased animals, be the causative agent of TSEs?

Further investigation determined that, although identical in amino acid sequence, the protein isolated from normal animals had biochemical and biophysical properties quite different from the one extracted from infected brains. While the structure of the normal form of the prion protein (PrP^{C}, for prion protein cellular) is mostly a-helical, the disease associated PrP^{Sc} is mostly B-sheet (Pan et al., 1993). This conformational difference is accompanied by other changes such as detergent solubility (PrP^{C} is soluble in non-denaturing detergents, PrP^{Sc} is not) and susceptibility to proteases (PrP^{C} is readily digested, whereas PrP^{Sc} is partially resistant) (Meyer et al., 1986; Oesch et al., 1985).

This data led to the now generally accepted 'protein-only' hypothesis that states that PrP^{Sc} is indeed the infectious agent, and that it is a modified, pathogenic form of its normal cellular counterpart, PrP^{C}. PrP^{Sc} is also proposed to 'replicate' by converting the normal isoform to the disease isoform by an auto-catalytic process(reviewed in (Prusiner, 1991)). This conversion seems to be limited to a conformational change, since no other differences in post-translational modifications have been detected (Prusiner, 1989). Much evidence has accumulated in favour of this hypothesis, including many transgenetic and gene targeting studies in mice (reviewed in (DeArmond and Prusiner, 1996; Scott et al., 1996)) and the cell-free, *in vitro* conversion of PrP^{C} to PrP^{Sc} (Kocisko et al., 1994).

Although the role of PrP^{Sc} in the mechanism of prion diseases has been the subject of much research, the function of PrP^{C} remains unknown. The examination of the biology of the normal isoform of the prion protein is the focus of this thesis and the remainder of this review.

Expression of the prion protein

PrP is encoded by a single copy gene, present in all vertebrates examined to date, and has been strongly conserved throughout evolution (Gabriel et al., 1992; Locht et al., 1986; Westaway and Prusiner, 1986). Human and murine PrP genes are located on chromosomes 20 and 2 respectively, which are homologous in the

two organisms (Sparkes et al., 1986). The genetic structure, extensively examined in hamster, has revealed that the entire PrP open reading frame is included within the second of two exons, which are separated by a 10 kb intron (Basler et al., 1986; Chesebro et al., 1985; Robakis et al., 1986). The immediate 5' region of the transcriptional start site does not have a TATA box, but contains GC-rich sequence clusters similar to those observed in several housekeeping genes (Basler et al., 1986).

Despite these promoter characteristics, expression of the PrP gene is both temporally and spatially regulated. In mouse embryos, PrP transcripts were detected, beginning at day 13.5, in developing brain, spinal cord and other non-neuronal tissues such as kidney and tooth buds. Additionally, PrP gene expression was observed in extra-embryonic tissues derived from the primitive ectoderm lineage such as the amnion, umbilical cord, and the mesodermal layer of the yolk sac (Manson et al., 1992). In newborn hamsters and rats, PrP gene expression has been observed in neurons, astrocytes and oligodendrocytes and was shown to increase two-fold during post-natal development (Moser et al., 1995).

Immunohistochemical analysis of protein expression in hamster organ preparations revealed that PrP^C is present in virtually all tissues examined. The protein was visualized, to varying degrees, in brain, heart, skeletal muscle, lung, stomach, intestine, adrenals, kidney, testis, ovary, spleen, pancreas, thyroid and thymus, but was undetectable in liver and erythrocytes (Bendheim et al., 1992).

Within the brain, PrP^C has a specific distribution and is concentrated in neurons and the neighbouring neuropil of gray matter, with very little immunostaining in the white matter (Bendheim et al., 1992). However, the lack of staining in white matter may be an artifact due to myelin, since prion mRNA has been detected in glial cells (Moser et al., 1995). The hippocampus contains the highest concentration of PrP^C, particularly in the stratum radiatum and stratum oriens of the CA1 region (Taraboulos et al., 1992). Cortex, fornix, septal nuclei, caudate, thalamus and brainstem also express substantial amounts of the protein.

This nearly ubiquitous expression of PrP in addition to its high degree of evolutionary conservation suggest that it plays an important role in normal cellular function.

Possible Function of the normal prion protein

Although its role in disease has been thoroughly characterized the physiological function of PrP has remained elusive. Its abundance on neurons has led to the proposal that it may function as a neural receptor or adhesion molecule and serve to direct and maintain the architecture of the nervous system (Hope and Manson, 1991). Others have suggested that PrP may be a proliferation factor for astrocytes, based on the dramatic gliosis observed in prion disease (DeArmond et al., 1987). Work from our own laboratory argues for a role in signal transduction, since antibodies to the protein blocked mitogen induced activation of lymphocytes (Cashman et al., 1990). This is in

accordance with the association of other GPI-linked molecules with defined signaling pathways. However, whether this is a general function of PrP^C or one specific to lymphocytes is unknown.

In the past several years, the generation of 'knockout' mice has yielded important information about the function of many molecules. Strikingly, mice homozygous for a null mutation of the PrP gene do not demonstrate any developmental or behavioural abnormalities (Bueler et al., 1992). Upon further investigation more subtle phenotypes were revealed. Electrophysiological studies demonstrated defective $GABA_A$ receptor-mediated fast inhibition and impaired long-term potentiation, suggesting that PrP^C may be required for normal synaptic transmission (Collinge et al., 1994). Altered circadian rhythms and loss of cerebellar Purkinje cells with advanced age have also been reported (Sakaguchi et al., 1996; Tobler et al., 1996). Similarities of some of these phenotypes with certain features of prion disease has implied that the loss of normal PrP^C function may contribute to neuropathology (Estibeiro, 1996).

Recently, an association of the N-terminal octarepeat region of PrP^C with copper has been reported. Examination of various tissues from PrP null mice revealed that brains of these animals contain approximately 20-fold less copper than those of wild type controls, whereas zinc and iron showed no significant difference (Brown et al., 1997a). Patch clamp analysis of Purkinje cells from PrP null mice demonstrate a significant reduction of inhibitory post-synaptic currents in the presence of copper (Brown et al., 1997b). Cerebellar

cells from these animals show an increased susceptibility to copper toxicity and are more sensitive to oxidative stress due to a decrease in copper/zinc superoxide dismutase-1 (SOD-1) enzyme activity (Brown et al., 1998; Brown et al., 1997). Release of PrP^C (as well as other GPI anchored proteins) from the surface of wild type cerebellar cells by phosphatidylinositol-specific phospholipase C (PIPLC) results in a reduction in the membrane copper content. These data strongly suggest that PrP^C is a major copper-binding protein of brain cells or that it behaves as a copper sensor, controlling the activity of other membrane-associated copper binding proteins. PrP^C may thus serve, directly or indirectly, to regulate the copper content of intracellular and extracellular compartments. Through this activity, PrP^C may therefore protect neurons from direct copper toxicity and from free radical damage by providing copper for enzymes such as SOD-1.

Does PrP have a receptor?

The cell surface localization of PrP^C, in addition to its proposed functions, make it likely that the protein interacts with other molecules on the same or adjacent cells. Indeed, it is becoming increasingly apparent that PrP^C may have a cognate receptor. The existence of such a molecule is suggested by Harris and colleagues in studies examining the recirculation of chicken PrP^C (chPrP).

Unlike many other GPI-linked proteins, chPrP was found to be closely associated with and endocytosed via clathrin coated pits

rather than caveolae (Shyng et al., 1994). Internalization by coated pits is dependent on interaction of the target protein with several intracellular components such as adaptor proteins. Since GPI-anchored proteins such as PrP lack a cytoplasmic domain, they cannot interact directly with the intracellular machinery of coated pits. PrP may therefore interact with a transmembrane protein which contains a coated pit localization signal to target the complex for endocytosis.

Additionally, N-terminal deletions of PrP^{C} reduced the localization to coated pits and the amount of protein endocytosed. The effect of the deletions was graded, with progressively larger deletions resulting in more pronounced reductions in the amount of protein internalized (Harris et al., 1996; Shyng et al., 1995), suggesting that this region of the protein is involved in the interaction with the proposed receptor.

Experiments with prion disease transmission in transgenic mice models have also led Prusiner and colleagues to postulate the existence of a prion interacting protein, which they have designated 'protein X' (Telling et al., 1995). Mice expressing both endogenous mouse PrP^{C} and transgenic human PrP^{C} proved to be resistant to disease induction by human prions. However, when these mice were crossed with others in which the mouse PrP gene had been ablated, the resulting animals were found to be susceptible to human prions.

These results led to the hypothesis that another molecule might be necessary for the formation of PrP^{Sc} and that this protein, much like

PrP^{Sc} itself, interacts with PrP^{C} most efficiently when the two proteins are from the same species. Thus, in mice expressing both mouse and human PrP, mouse protein X is bound to mouse PrP and therefore unavailable to participate in the formation of human PrP^{Sc}. Only upon removal of mouse PrP will mouse protein X be available for binding to human PrP, making these animals susceptible to human prions. Further transgenic analyses using various point mutations in PrP^{C} have mapped the interaction site of protein X to a discontinuous epitope with in the C-terminus (Kaneko et al., 1997).

Although protein X may be more of a co-factor or chaperone than a true receptor, it is nonetheless a prion binding protein. It is interesting to note that the interaction site of protein X is different from that proposed by Harris and colleagues for their receptor, suggesting that these may be two unrelated proteins with potentially different functions.

The direct search for PrP^{C} binding molecules was pioneered by Oesch and colleagues, who have probed "ligand blots" of brain extracts with PrP^{Sc}, or with short synthetic peptides derived from PrP sequence (Oesch et al., 1990). Of three PrP ligands (Plis) reported to date, the most abundant is glial fibrillary associated protein (GFAP) (Telling et al., 1995). Another Pli has been identified as PSF, a splice factor associated with the polypyrimidine-tract binding protein, and the third is not an intrinsic membrane protein (Oesch, 1994). It is unlikely that the Oesch PrP^{C} ligands have normal biological

relevance, considering that PrP^C is expressed at the cell surface and none of the identified proteins are integral membrane components.

Recently, two reports have claimed the identification of a receptor for PrP. A complementary hydropathy strategy was used to design a peptide that would hypothetically mimic a PrP receptor binding site (Martins et al., 1997). Antibodies raised against this peptide recognize an unknown protein of 66 kDa present on neurons. Bacterially produced PrP was indirectly shown to interact with a protein recognized by this antibody.

In a more classical approach, Rieger and colleagues used a yeast two-hybrid screen to identify PrP^c interacting proteins, one of which was the 37 kDa laminin receptor precursor (LRP) (Rieger et al., 1997). The LRP-PrP interaction was confirmed using proteins produced in both insect and mammalian cells. Interestingly, the levels of LRP were increased in the brains and spleens of scrapie infected mice relative to control animals, indicating a potential interaction with PrP^{Sc} as well as PrP^C.

The sheer difference in size of these two candidate proteins indicates that they are probably not the same. Since neither study provides a measure of the affinity of the proposed interaction or an indication of the functional domains involved, a more detailed comparison cannot be made. The tissue distributions of these proposed receptors does not entirely correlate with that of PrP^C, which would be expected of a cognate receptor, and no functional basis for these interactions is

proposed. Thus, the lack of 'incriminating' data makes it impossible to determine whether either one of these molecules represent a true functional, high affinity receptor for the normal cellular isoform of the prion protein. However, it is clear that PrP^C can interact with other cell surface proteins and that some or all of these interactions may be relevant for the normal function of PrP^C.

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Chapter 2

Prion Protein Expression in Human Leukocyte Differentiation

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Prion Protein Expression in Human Leukocyte Differentiation

By Vincent C. Dodelet and Neil R. Cashman

The cellular isoform of the prion protein (PrP^C) is a small glycoprotein attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol anchor. This molecule is involved in the pathogenesis of prion diseases in both humans and animals. We have characterized the expression patterns of PrP^C during human leukocyte maturation by flow cytometry with monoclonal antibodies to PrP^C, the glycan moiety CD15, and the stem cell marker CD34. We observe that prion protein is present on $CD34^+$ bone marrow (BM) stem cells. Although lymphocytes and monocytes maintain PrP^C expression throughout their differentiation, PrP^C is downregulated upon differentiation along the granulocyte lineage. In vitro retinoic acid–induced differentiation of the premyeloid line HL-60 into granulocyte-like cells mimics the suppression of PrP^C in granulocyte differentiation, as both PrP^C mRNA and protein are downregulated. These data suggest that selected BM cells and peripheral mononuclear cells may support prion agent replication, because this process is dependent on availability of PrP^C. Additionally, retinoic acid–induced extinction of PrP^C expression in HL-60 cells provides a potential model to study PrP gene regulation and protein function. Finally, these data suggest the existence of cell-specific glycoforms of PrP^C that may determine cellular susceptibility to infection by the prion agent.

THE PRION PROTEIN is best known for its involvement in the transmissible spongiform encephalopathies (TSE) or prion diseases, a group of human and animal neurodegenerative diseases which include Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle. The pathology of the TSEs is characterized by neuronal loss, spongiform change, gliosis, and accumulation of an abnormal protease-resistant protein designated PrP^{Sc} in both brain and peripheral tissues, including organs of the lymphoreticular system. The prevalent "protein-only" hypothesis of prion disease proposes that PrP^{Sc} is indeed the infectious agent, and that this modified, pathogenic form of the normal cellular protein replicates by converting the normal isoform to the disease isoform.[1]

The normal cellular isoform of the prion protein PrP^C is a small glycosylphosphatidylinositol (GPI)-anchored cell-surface protein expressed predominantly in the brain, but also in a wide variety of peripheral tissues, including peripheral mononuclear blood cells.[2,3] PrP^C is present in all vertebrates examined to date, is widely expressed during embryogenesis, and has been strongly conserved throughout evolution, suggesting a vital function for the protein.[4-6] Surprisingly, PrP null mice have shown that the protein is apparently not necessary for normal murine development or mature survival because these mice are viable, do not show any obvious developmental phenotype, and show no behavioral abnormalities up to 93 weeks of age.[7,8] Ablation of the prion gene renders these mice resistant to experimental scrapie and obviates accumulation of PrP^{Sc}.[8] More target-directed assays have shown subtle abnormalities in null mice such as defective $GABA_A$ receptor-mediated fast inhibition and impaired long-term potentiation, altered circadian rhythms, and loss of cerebellar Purkinje cells.[9-11] These phenotypes are reminiscent of certain features of TSEs and suggest that loss of the normal function of this protein may contribute to disease.[12]

Clues to the function of PrP^C may be gleaned by examination of cell-specific expression patterns. In peripheral blood of humans and rodents, PrP^C is detected at the cell surface of lymphocytes and monocytes, but is absent from erythrocytes and granulocytes.[2,3] Our laboratory has previously determined that PrP^C expressed by mononuclear cells may play a role in cell activation.[2] In this study, we further characterized the expression patterns and gene regulation of the protein in cells of the immune system. We now show that PrP^C is expressed very early in hematopoiesis, indicated by its presence on $CD34^+$ stem cells. Differentiation along the lymphocyte or monocyte lineages supports the expression of surface PrP^C, while differentiation along the granulocyte lineage progresses with concomitant downregulation of PrP^C surface protein. This cell-type specific extinction of PrP^C in granulocyte differentiation can be recapitulated in vitro by retinoic acid induction of HL-60 cells.

These results allow us to better define the regulation of the expression of PrP^C in human leukocytes, which has potential implications for human and animal prion diseases. In addition, our studies provide a novel in vitro system with which to study PrP gene regulation and protein function.

MATERIALS AND METHODS

Cells. Human peripheral blood leukocytes were isolated from whole blood from healthy volunteer donors and separated using Lymphocyte-poly (Cedarlane, Hornby, Ontario, Canada) density centrifugation. The two resulting layers were pooled, washed three times with phosphate-buffered saline (PBS), and stained for flow cytometry analysis as described in the following section.

Human low-density bone marrow (BM) cells were isolated by Ficoll-Paque (Pharmacia, Baie D'Urfé, Québec, Canada) density centrifugation of BM aspirates from patients undergoing diagnostic BM evaluation (kindly provided by Dr Alan Brox, Royal Victoria Hospital,

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McGill University). Cells were washed extensively and stained for flow cytometry analysis.

The human premyeloid line HL-60 (ATCC CCL-240) was maintained in RPMI 1640 medium (GIBCO-BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS), 2.5 mg/mL penicillin, 2.5 mg/mL streptomycin, and 2 mmol/L glutamine. Differentiation along the monocytoid lineage was induced by culture in 100 nmol/L of phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO) for 3 days. Differentiation along the granulocyte lineage was induced by culture in 1 mol/L all-*trans* retinoic acid (Sigma) for 0, 1, 3, and 6 days. Cells were then stained for flow cytometry analysis or procured for isolation of total RNA.

Monoclonal antibodies (MoAbs) and flow cytometry. The MoAb 3F4, which uniquely recognizes PrP^C at the cell surface[2,13] (and N.R.C., unpublished data), was prepared as ascites fluid in pristane-primed Balb/c mice, and used unconjugated at a 1:5,000 dilution. 3F4 was followed by either a goat anti-mouse Fab fluorescein isothiocyanate (FITC) conjugate or a donkey anti-mouse phycoerhythrin (PE) conjugate (both from Jackson Immunoresearch, West Grove, PA). Anti–CD15-FITC and anti–CD34-PE MoAb direct conjugates were obtained from Becton Dickinson (Saint-Laurent, Québec, Canada). FITC-conjugated, PE-conjugated, and unconjugated isotype-matched irrelevant control MoAbs were included in all experiments (Becton Dickinson and Sigma).

For immunofluorescence staining, cells were first incubated on ice for 30 minutes in PBS supplemented with 10% normal goat serum to block nonspecific binding. Cells were then stained with the appropriate MoAbs, each for 30 minutes on ice. Samples were analyzed on a FACSCAN flow cytometer using LYSYS II software (Becton Dickinson).

Semiquantitative polymerase chain reaction (PCR) analysis. HL-60 cells were differentiated with all-*trans* retinoic acid as described. Cells were harvested at days 0, 1, and 3 and total RNA was isolated using TRIzol (GIBCO-BRL). Total RNA was then treated with RQ1 RNase free DNase (Promega, Madison, WI) and re-extracted twice with phenol-chloroform. Total RNA was reverse-transcribed to cDNA using Superscript reverse transcriptase (GIBCO-BRL) according to the manufacturer's instructions. This cDNA was then used for both prion and β-actin amplification by PCR. Primers used for the PCR reactions had the following sequences: human prion forward 5'-AAGCCTGGAGGATGGAACACT-3', reverse 5'-GTTGCTGTACTCATCCATGGG-3', and β-actin forward 5'-ATGCCATCCTGCGTCTGGACCTGGC-3', reverse 5'-AGCATTTGCGGTGCACGATGGAGGG-3'. The primers for human prion and β-actin were designed to generate fragments of 434 and 606 bp, respectively. Two hundred nanograms of cDNA was added to the reaction mixture containing PCR buffer, 0.5 mmol/L dNTPs (Pharmacia), 50 pmol of either primer set, and 0.5 μL Taq polymerase (GIBCO). Samples were placed in a thermocycler (Perkin Elmer Cetus Corp, Norwalk, CT) for 25 cycles of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. The PCR products were separated on a 1% 1× Tris-acetate EDTA (TAE) agarose gel, transferred to a nylon membrane overnight by capillary action, and the Southern blots were probed with the appropriate random primed [^{32}]P-labeled probes for 16 hours at 42°C in Rapid-Hyb buffer (Amersham, Burlington, Ontario, Canada). Blots were washed, and exposed to Phosphor Screen and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Prion protein expression in human peripheral blood cells. Human peripheral blood leukocytes were analyzed for PrP^C surface immunoreactivity by flow cytometry (Fig 1). Three main populations can be defined on the basis of size (forward scatter [FSC]) and granularity (side scatter [SSC]). When these three populations are examined for PrP^C and CD15 immunoreactivity, each presents a unique staining profile. CD15 provides a convenient secondary marker because of its differential distribution among leukocytes.[14-16] Lymphocytes have low to intermediate levels of PrP^C and are $CD15^-$, monocytes are PrP^C high and CD15 low, and granulocytes are PrP^{C-} and CD15 high. Erythrocytes are negative for both markers (data not shown). The absence of PrP^C on granulocytes suggests at least two possibilities: lymphocytes and monocytes acquire PrP^C during differentiation, or granulocytes lose PrP^C as they mature.

Prion protein expression in human BM cells. To examine the question of acquisition or suppression of PrP^C in leukocyte differentiation, we first examined PrP^C expression in $CD34^+$ multipotential stem cells. As shown in Fig 2, cells that express CD34 coexpress surface prion protein. This suggests that prion protein may indeed be specifically downregulated in the granulocyte and erythroid lineage, and that other lineages maintain their expression of prion protein. We further investigated this possibility by analyzing low density human BM cells by flow cytometry (Fig 3). Cells that are differentiating along the granulocyte lineage show a dramatic increase in CD15 staining in the maturation process. These highly granular cells show a progressive loss of PrP^C surface immunoreactivity as they gain CD15 staining. These data demonstrate that prion protein is present on pluripotential stem cells, and that it is downregulated during subsequent differentiation along the granulocyte lineage.

Regulation of prion protein expression in differentiating HL-60 cells. To provide a more convenient model of myeloid cellular differentiation, we examined the premyeloid cell line HL-60, which can be induced to differentiate along either the granulocyte or monocyte lineages by retinoic acid or phorbol esters, respectively.[17-20] HL-60 cells express PrP^C, which persists upon phorbol ester induced differentiation into macrophage-like cells (Fig 4). Conversely, induction of HL-60 cells into granulocyte differentiation by retinoic acid was associated with progressive downregulation of surface PrP^C (Fig 5). The cells were observed over a 6-day period, with the proportionally largest decline in surface prion protein immunoreactivity occurring after the first 24 hours (Fig 5B).

To investigate the role of gene transcription in the loss of granulocyte PrP^C immunoreactivity, we examined the level of mRNA for the prion protein by semiquantitative PCR. To exclude potential contamination by PrP genomic sequences, the total RNA extract was treated with RNase-free DNase before PCR amplification. HL-60 cells were differentiated with retinoic acid as described and harvested for RNA extraction after 24 and 72 hours. A progressive decrease in the level of prion mRNA can be observed, while the level of β-actin mRNA remains approximately constant (Fig 6). This shows a strong association between decreased surface PrP^C immunoreactivity and decreased PrP mRNA, most probably the result of decreased transcription triggered by retinoic acid treatment.

DISCUSSION

The normal cellular isoform of the prion protein, PrP^C, is highly expressed in brain as well as many peripheral tissues,

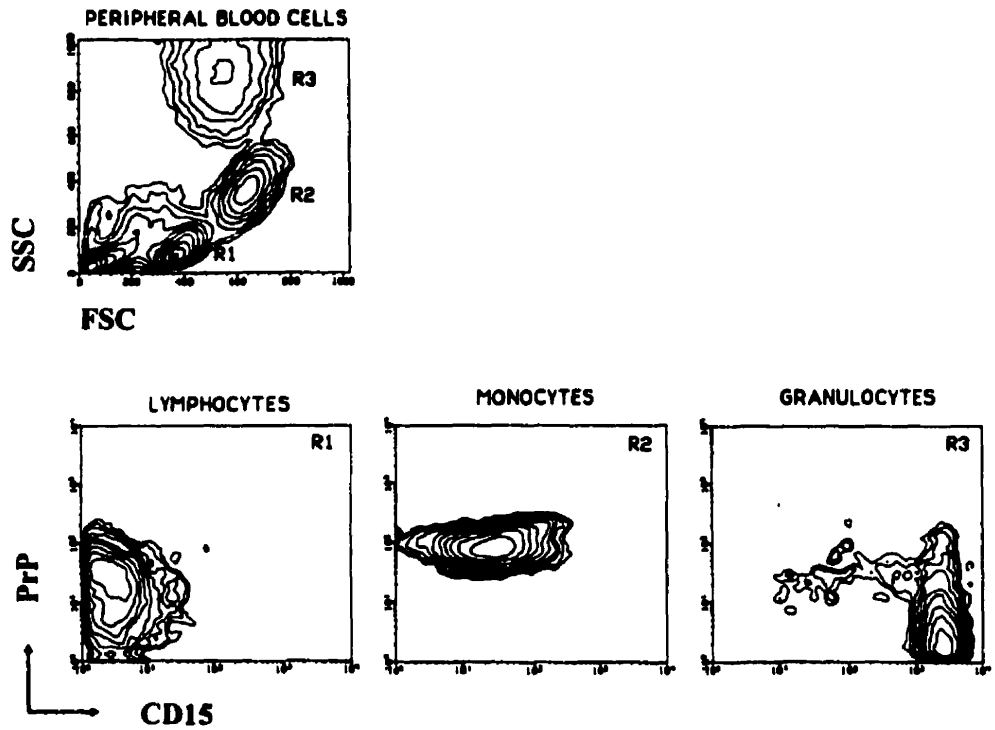


Fig 1. Expression of prion protein versus CD15 by human peripheral blood leukocytes. Leukocytes were isolated from whole blood of normal donors, washed, stained for PrP^C and CD15, and analyzed by flow cytometry. A typical light scatter contour plot of normal peripheral human blood mononuclear cells is shown in the upper left. Analysis gates R1-R3 are set on forward and side scatter alone and define three major subpopulations: lymphocytes (R1), monocytes (R2), and granulocytes (R3). Contour plots of surface prion protein versus CD15 for each of the three subpopulations are shown. Lymphocytes are (PrP^+, $CD15^-$), monocytes are (PrP^+, $CD15^{low-med}$), and granulocytes are (PrP^-, $CD15^{hi}$). The small amount of PrP^+ cells in panel R3 are due to contaminating monocytes.

including peripheral blood mononuclear cells. We have found that PrP^C is expressed by $CD34^+$ multipotential stem cells in human BM. PrP^C is downregulated in stem cell differentiation to the granulocyte lineage, but is maintained in lymphoid and monocyte lineages. The downregulation of PrP^C in myeloid differentiation, at both the protein and mRNA levels, was modeled in vitro by the induction of HL-60 cells to granulocytoid lineage by retinoic acid. Our findings differ from Diomede et al,[21] who reported PrP^C expression in neutrophils, as well as lymphocytes and monocytes. However, these studies were based on cell populations of 95% purity, in which contamination by mononuclear cells may have contributed to the PrP mRNA and protein detected by PCR and immunoprecipitation, respectively. It is also at least formally possible that PrP^C is a cell-surface molecule in mononuclear cells detectable by flow cytometry, and an internally sequestered protein in neutrophils.

The regulation of expression of PrP^C is incompletely understood. Despite the tissue-regulated and developmentally regulated expression of PrP^C, the immediate 5' region of the transcriptional start site does not contain a TATA box, but displays a GC-rich sequence similar to that observed in many housekeeping genes.[22] Enhancer elements have been mapped upstream of the start site, and are also apparently present in the long first intron (~10 kb) detected in all vertebrate species whose genomic PrP^C organization is known.[23,24] Our study suggests that retinoic acid–responsive elements participate in the cell-specific regulation of PrP^C, and provides a biologically relevant in vitro paradigm to investigate the molecular basis of this regulation. Alternately, retinoic acid may be inducing the expression of *trans*-acting proteins that act at a silencing element controlling PrP expression. The regulation of prion protein expression in granulocytes and erythrocytes may underlie a general regulatory mechanism that could be exploited to reduce the amount of PrP^C available for conversion in the prion diseases, because it has been shown that endogenous expression of PrP is necessary for continued propagation of the scrapie agent.[8]

Prion infectivity apparently copurifies with PrP^{Sc}, the protease resistant isoform of PrP^C.[25,26] Cell-free conversion of PrP^C to PrP^{Sc} has now been accomplished in vitro.[27] Replication of the prion agent appears to be strictly dependent on availability of PrP^C, because mice homozygous for the targeted disruption of the PrP gene are completely resistant to scrapie, and heterozygous mice display a longer incubation and slower disease progression than wild-type mice.[8] Thus, the distribution of PrP^C in hematogenous cells may predict the capability of that

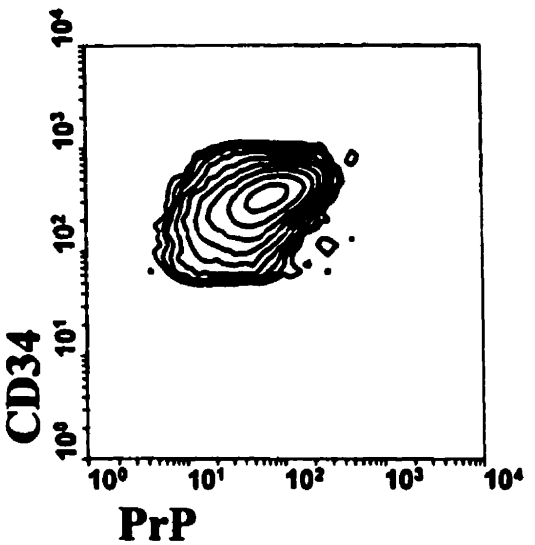


Fig 2. Surface prion protein expression by human CD34+ BM cells. Human BM aspirates were fractionated for low-density BM cells to enrich for multipotential stem cells. Cells were stained for CD34 and PrP^C and analyzed by flow cytometry. Contour plot of low-density human BM cells shows that all CD34+ cells also express PrP^C. Data were analyzed using gates for both high side scatter and CD34 expression.

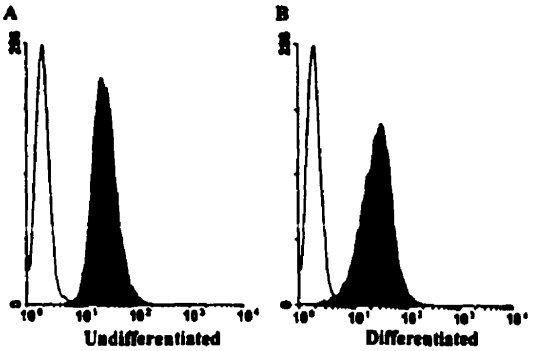
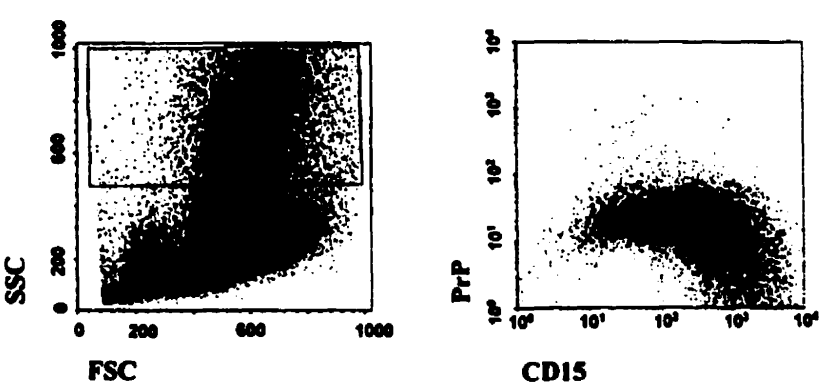


Fig 4. Surface prion protein expression by HL-60 cells persists upon induction of monocyte lineage differentiation. HL-60 cells were cultured in the presence or absence of 100 nmol/L PMA for 3 days, then stained for flow cytometry analysis with MoAb 3F4. Histograms show PrP^C surface immunoreactivity relative to an IgG2b isotype control. Minimal change is observed in PrP^C staining intensity between undifferentiated (A) and differentiated cells (B).

cell to propagate and/or transmit prion infectivity in hosts transfused or transplanted with those cells. On the basis of our current report, BM cells, including $CD34^+$ stem cells, might be expected to harbor the prion agent, despite current guidelines which do not recognize BM as high-risk tissue.[28,29] In the periphery, monocytes and lymphocytes, which express PrP^C, would be expected to support prion replication, whereas PrP^C negative erythrocytes and granulocytes would not. This contention is supported by a limited number of transmission experiments[30,31] and by recent data from Blattler et al,[32] who show that prion agent infectivity detected in spleen by bioassay is dependent on the expression of PrP^C by spleen cells and does not accumulate by nonspecific "carry-over" from the original innoculum.

An additional implication of our study is that PrP^C must be differentially glycosylated in the nervous system as opposed to the periphery. PrP^{Sc}, the abnormal disease-associated isoform of PrP^C, has been found to possess a high proportion of N-linked glycan chains terminating in the glycosyl moiety stage-specific embryonic antigen 1 (SSEA-1), also known as CD15.[33] This carbohydrate moiety has been found to participate in cell-cell adhesion and tissue differentiation early in development, and probably plays an important role in immunocyte adhesion in inflammatory processes.[34,35] In the peripheral blood, granulocytes, which express no PrP^C, display prominent immunoreactivity for CD15. Lymphocytes, which display no surface CD15, do express PrP^C. Only peripheral monocytes possess surface immunoreactivity for both PrP^C and CD15, allowing the possibility that monocyte PrP^C may be modified with the SSEA-1 moiety. Because glycosylation may participate in the brain-region-specific replication of the scrapie agent,[36] it is possible that certain PrP^C on different peripheral cells might be more likely to participate in scrapie infection. Of interest, some data indicate that the monocyte-macrophage cell lineage is critical to the propagation of scrapie infectivity in the periphery. Scrapie infection in severe combined immunodeficient mice is apparently dependent on dendritic cells,[37] which are tissue

Fig 3. Downregulation of surface prion protein during in vivo granulocyte maturation. Human BM aspirates were fractionated for low-density BM cells as described. Cells were washed and stained for flow cytometry analysis as described in the legend to Fig 1. Differentiating granulocytes displaying high side scatter were gated as shown on left. As these cells mature along the granulocyte lineage and become $CD15^{hi}$, there is a progressive loss of surface PrP^C staining.



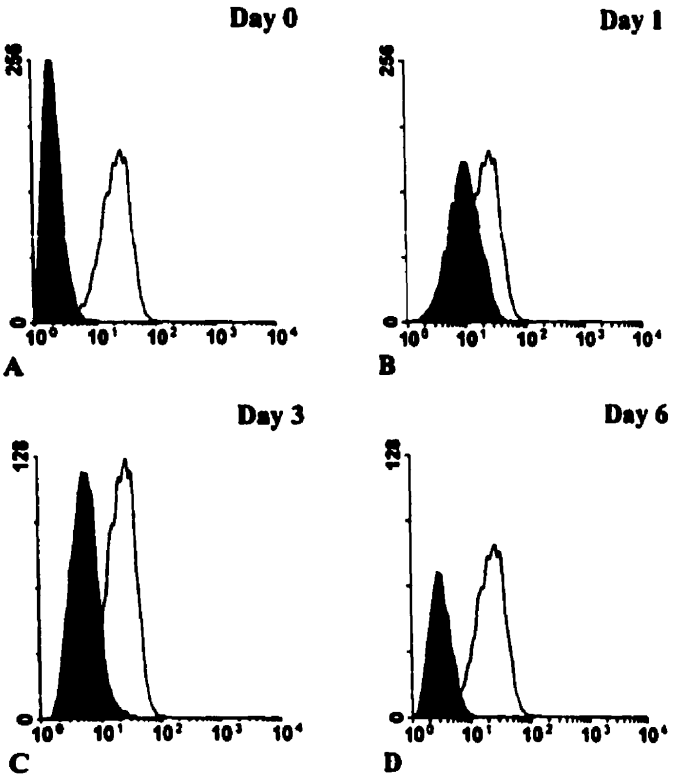


Fig 5. Downregulation of surface PrP^C in retinoic acid-induced granulocytic differentiation of HL-60 cells. HL-60 cells were cultured in the presence of 1 mol/L all-trans retinoic acid for 0, 1, 3, and 6 days, then stained for flow cytometry analysis with MoAb 3F4. Basal level of PrP^C staining in undifferentiated cells (solid line) is shown relative to an IgG2b isotype control (in gray) (A). The progressive decrease of PrP^C staining during differentiation (in black) is shown relative to basal level (B through D).

macrophages specialized for immune presentation. Moreover, peripheral blood cells containing scrapie infectivity are relatively radiation insensitive,[38] which is consistent with the end-mitotic nature of peripheral monocytes. Finally, monocytes may be unique among peripheral blood cells for their capability of processing PrP^C to PrP^{Sc}.[39] Because other peripheral blood cells also express PrP^C, the capability of monocytoid cells to support scrapie agent replication may relate to a posttranslational modification, such as SSEA-1 modification of the terminal PrP^C glycans.

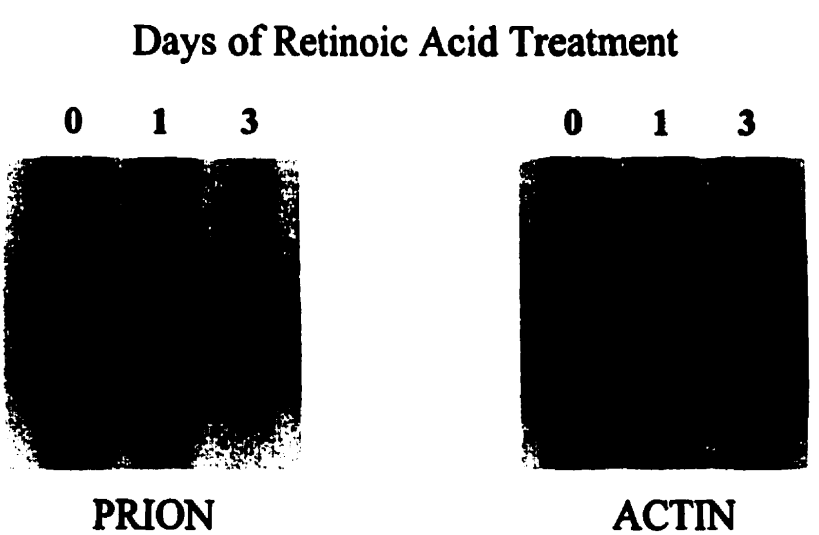


Fig 6. Downregulation of PrP mRNA in retinoic acid-induced differentiation of HL-60 cells. HL-60 cells were cultured in the presence of 1 mol/L all-trans retinoic and obtained at days 0, 1, and 3. Total RNA was isolated and reverse-transcribed to cDNA, which was then used for both prion and β-actin amplification by PCR. The PCR products were then Southern blotted with radioactively labeled PrP and β-actin probes. Blots were analyzed by Phosphorimager scanning.

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Following our investigation of prion protein expression in human leukocyte differentiation, we shifted our focus to potential functions of this highly regulated protein. We chose to examine the possible existence of a cell surface receptor for PrP^C using soluble, recombinant versions of the protein. This chapter outlines the design, production and initial characterization of the functionality of these prion protein constructs.

Chapter 3

Generation of soluble prion protein constructs

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Summary

Prions are best known for their involvement in scrapie, bovine spongiform encephalopathies (BSE), and Creutzfeldt-Jakob disease. The pathogenic, protease-resistant form (PrP^{Sc}), believed to be the causative agent of the prion diseases, is derived from the normal cellular isoform of the protein (PrP^{c}). Little is known of the physiological role of PrP^{c}, although it has been shown that it is a necessary precursor of PrP^{Sc} as demonstrated by the resistance to disease induction of PrP knockout mice. Previous work from our laboratory has determined a role for PrP^{c} in lymphocyte signaling, and has led to our hypothesis that normal cellular prion protein may be a receptor with one or more cognate ligands. We report here the development of several soluble recombinant forms of PrP^{c} as protein probes for ligand screening. A prion protein-alkaline phosphatase fusion construct has been used successfully to demonstrate a PrP dependent binding activity on the surface of several cell lines.

Introduction

It is likely that a better understanding of the function of the normal cellular isoform of the prion protein will provide important clues to its subversion in prion disease. However, the function of PrP^c in normal cells is unknown, and its protein sequence is not significantly related to any other protein in the databases, disallowing any functional analogies (Prusiner, 1991).

Our laboratory has approached the problem of PrP^c function by discovering and characterizing its role in lymphocyte activation (Cashman et al., 1990). It was found that lymphocyte PrP^c was dramatically upregulated by cell activation, and that antibodies directed against PrP^c suppressed T cell activation induced by the lectin mitogen concanavalin A. These results place PrP^c in the context of other cell surface glycosylphosphatidylinositol (GPI)-linked proteins such as decay accelerating factor (CD55), Thy1, CD59, and Qa-2 which have been shown to modulate T cell mitogenesis (Cook et al., 1992; Davis et al., 1988; Korty et al., 1991; Pont et al., 1985). The signal-transducing function of PrP^c is not limited to lymphocytes. A recent study of transplantation of normal mouse brain tissue into PrP -/- null mice demonstrated that cell surface PrP^c is essential for neurodegeneration and spongiform change in the transplanted tissue (Brandner et al., 1996). These data, implicating PrP^c in cellular activities as important as lymphocyte activation and neuronal death, strongly suggest that PrP^c can function in signaling local environmental changes -- in other words, a receptor. One would

anticipate that a functionally important cell surface receptor such as PrP^c must have a cognate ligand.

The direct search for PrP^c binding molecules was pioneered by Oesch and colleagues, who have probed "ligand blots" of brain extracts with PrP^{Sc}, or with short synthetic peptides derived from PrP sequence (Oesch et al., 1990). Of three PrP ligands (Plis) reported to date, the most abundant is glial fibrillary associated protein (GFAP) (Telling et al., 1995). Another Pli has been identified as PSF, a splice factor associated with the polypyrimidine-tract binding protein, and the third is not an intrinsic membrane protein (Oesch, 1994; Oesch et al., 1995). It is unlikely that the Oesch PrP^c ligands have normal biological relevance, considering that PrP^c is expressed at the cell surface.

Recently, other techniques have been used to identify candidate ligands for PrP^c. Martins *et al* have used a complementary hydropathy approach to generate an antibody which recognizes a 66 kDa protein on the surface of neurons (Martins et al., 1997). Rieger *et al* have used a more conventional yeast two-hybrid screen to identify the 37 kDa laminin receptor precursor as a potential PrP^c binding protein (Rieger et al., 1997). Molecular weight alone suggest that these two proteins are most probably different and the lack of physiological data concerning them makes it difficult at this time to determine their validity as candidate ligands. It is also quite possible that more than one ligand exists.

We decided to approach the question of the existence of a PrP^c ligand using a technique that would maximize relevant cell surface interactions. By using recombinant soluble forms of the prion protein as protein probes, cell lines and tissues can be screened for the presence of one or several cell surface binding proteins. Additionally, we chose to use mammalian production systems for these recombinant proteins to ensure proper folding and glycosylation of PrP^c. This chapter details our efforts to construct and express such reagents with PrP^c. Three different constructs were generated: A truncated PrP^c, lacking the GPI-anchor (PrPΔ); a fusion of PrP^c and the constant portion of human IgG1 heavy chain (PrP-Ig); and a fusion of PrP^c and the heat-stable human placental alkaline phosphatase (PrP-AP).

Results

Truncated PrP

It had been previously demonstrated that deletion of the GPI anchor signal sequence results in secretion of these proteins and truncated soluble versions of cellular PrP^c have been detected in protein extracts of both human platelets and hamster brain (Borchelt et al., 1993; Caras and Weddell, 1989; Perini et al., 1996). Such a soluble form of PrP^c would be minimally modified, ensuring proper folding and post-translational modification and could be detected by direct radioactive labeling, or indirectly with anti-PrP antibodies. A construct was therefore generated that was truncated after the Ser^{230} codon (PrPΔ), thus completely lacking the GPI anchor signal sequence (Figure 3.1). Although some protein was produced in transient transfection assays of COS-7 cells (data not shown), this proved to be too little for functional assays. Additionally, the half-life of this truncated form of PrP^c was very short in solution, as has been shown by others (Borchelt et al., 1993).

Given the difficulty of production and short half-life of PrPΔ, work with this construct was not pursued. We surmised that development of a fusion protein with a stable non-PrP domain might prevent PrP degradation and allow for high level production. The availability of a defined protein tag would also aid in purification and detection of a PrP fusion protein.

Fusion proteins

PrP-Ig

In order to generate large amounts of a stable soluble form of PrP^c with which to screen binding to cell lines and tissues, we designed a fusion protein consisting of the entire open reading frame of human PrP^c up to and including Ser^{230} (thus excluding the GPI anchor sequence) linked in frame to the hinge, CH2, and CH3 domains of human immunoglobulin G1 (IgG1) heavy chain (Figure 3.2). Such an Ig fusion provides many advantages over untagged soluble constructs. Simple purification and detection procedures are possible with such constructs, and the covalently bound dimer form may increase affinity of interaction by clustering and cooperative binding. The chimeric protein was successfully expressed by transient transfection of COS-7 cells and was shown to migrate as a dimer under non-denaturing SDS-PAGE conditions (Figure 3.3 A, B).

Unlike the PrPΔ construct, μg amounts of PrP-Ig were readily produced and the fusion protein appeared to be stable and was easily purified. The fusion protein was used for binding assays with flow cytometry either directly in supernatant form or as a purified disulfide linked homodimer. Analysis of a multitude of primary cells and cell lines was carried out, but we could not identify cells that were positive for expression of surface PrP ligands. More sensitive, radioactive assays using either ^{35}S-methionine labeled PrP-Ig or detection using protein-A ^{125}I also failed to demonstrate the presence of a cell surface ligand. This recombinant soluble form of PrP may have been particularly susceptible to proteolysis since growth of transfected cells in serum free medium yielded a protein

product that corresponded to only the Ig portion of the fusion protein, (Figure 3.3 C). PrP-Ig may have been cleaved by cellular proteases normally held in check by protease inhibitors present in serum.

PrP-AP

Our third attempt at generation of a recombinant soluble form of the prion protein was made using the secreted human placental alkaline phosphatase (AP) fusion protein partner pioneered by Drs. J.G. Flanagan and P. Leder (Flanagan et al., 1991). The AP provides a practical tag to which commercially available antibodies are available. In addition, the enzymatic activity of AP can be detected using simple chromogenic assays using a variety of substrates without the need for purification or labeling. Sensitivity using AP fusion proteins has been shown to be comparable to purified and radioactively labeled reagents (Flanagan et al., 1991; Flanagan and Leder, 1990).

In order to generate a PrP-AP fusion construct, the cDNA for mouse prion (up to and including the Arg^{229} codon) was ligated in frame into the APtag-2 expression vector, which has an SV40 origin of replication and a CMV promoter, allowing for vector amplification and high level fusion protein production in COS cells. As a negative control, secreted human placental AP with its own leader sequence (SEAP) was expressed from the pCDNA1 vector at levels similar to that of the fusion protein (Figure 3.4).

Consistent with expectations from the design of these vectors, PrP-AP transfected COS cells secreted a 97 kD protein which is immunoreactive with both AP and PrP antibodies. CMV/SEAP-transfected COS cells express a 67 kD protein which is immunoreactive with an AP monoclonal antibody, but not PrP polyclonal antibodies (Figure 3.5). Both PrP-AP and AP are expressed and secreted by COS cells at 5-10 μg protein per ml supernatant, and display similar supernatant AP activity. Supernatants of untransfected or mock-transfected COS cells do not contain detectable AP activity or immunodetectable PrP^c.

Cell lines of neuronal and fibroblast origin were tested for the presence of PrP^c binding proteins using a simple chromogenic adhesion assay. Cells were incubated with conditioned media containing SEAP or PrP-AP, washed and lysed. Lysates were then heated at 65°C to inactivate the endogenous cellular alkaline phosphatases and the lysates assayed for bound AP activity. Incubation with SEAP resulted in minimal background levels as compared to the PrP-AP. The cell lines tested were found to be positive for PrP-AP binding, but to varying degrees, indicating a variable level of expression of the ligand(s) among the different cell lines (Figure 3.6).

Our data demonstrate that we have successfully constructed and expressed a PrP-AP "affinity reagent" with which to seek distribution, function and molecular identity of the PrP^c ligand.

Discussion

To seek the PrP^c cell surface ligand, we have generated and expressed three different PrP constructs: a truncated PrP^c, lacking the GPI-anchor (PrPΔ); a fusion of PrP^c and the constant portion of human IgG1 heavy chain (PrP-Ig); and a fusion of PrP^c and the heat-stable human placental alkaline phosphatase (PrP-AP). The PrPΔ construct proved to be too difficult to produce in useful quantities. The PrP-Ig construct was secreted efficiently by transfected cells, but did not show detectable binding activity. Only the PrP-AP construct proved useful in our attempt to identify a PrP^c ligand. This construct is efficiently secreted from transfected cells in high quantities, appears stable, and is readily usable as crude conditioned media.

Our decision to use a mammalian expression system for the generation of recombinant PrP^c constructs was guided by the successful generation of a large variety of similar constructs for use in ligand detection. CD44, P-selectin, c-kit, Fas, Mek4 and Sek are just some of the cell surface receptors that have been used as either Ig or AP fusion proteins to identify their previously unknown ligands (Aruffo et al., 1990; Cheng and Flanagan, 1994; Flanagan and Leder, 1990; Sako et al., 1993; Suda et al., 1993). Although bacterial systems would have provided a simpler alternative, several reports stated problems in attempting to produce full length PrP^c in bacterial systems (Hornemann et al., 1997; Swietnicki et al., 1997; Zahn et al., 1997; Zhang et al., 1997). Full length PrP^c has been shown to

accumulate in inclusion bodies, requiring the use of harsh detergents and denaturants for purification. The protein then has to be renatured and refolded accurately, adding another level of uncertainty to this method of production. However, several features of a eukaryotic expression system would favour the normal folding and post-translational processing of a recombinant PrP^c. Glycosylation was of particular concern since mutation of the two sites of attachment of N-linked glycans results in intracellular sequestration of PrP^c, suggesting an important function in proper folding and transport of the molecule (Lehmann and Harris, 1997; Rogers et al., 1990).

Use of eukaryotic production systems did not guarantee success however, as two out of three constructs proved to be either difficult to produce or non-functional when produced. Deletion of the GPI anchor sequence or attachment of the Ig tag may have affected proper folding of the proteins and/or interfered with appropriate glycosylation. Additionally, our observations with regards to the PrPΔ and PrP-Ig constructs suggest that recombinant, soluble forms of the protein may be susceptible to degradation. The N-terminus of PrP has been implicated in binding of other molecules as suggested by the accumulation of N-terminal deleted PrP^c at the cell surface (Harris et al., 1993). The N-terminus of PrP is rich in basic amino acids, which could serve as a substrate for dibasic peptidases, secreted from the cell or present in serum. If the N-terminus were involved in binding of PrP^c to its ligand, then loss of this region would ablate binding. This may explain the lack of binding activity observed for PrP-Ig relative to PrP-AP, although it is unclear why

one construct would be more prone to proteolysis than the other. A more detailed analysis of the involvement of the N-terminus of PrP^c in ligand binding is presented in the following chapter.

Use of PrP-AP in the identification of a number of cell lines exhibiting a PrP^c binding activity will allow us to proceed with the characterization and identification of the ligand(s). This work is detailed in the following chapters.

Materials & Methods

Constructs: PrPΔ

A 714 bp fragment encoding the human PrP open reading frame (ORF) up to and including Ser^{230} was amplified from human peripheral blood leukocyte (PBL) RNA using RT-PCR. Human PBL were isolated from whole blood from a healthy donor (VD) by Ficoll-Paque (Pharmacia, Baie D'urfé, QC) density centrifugation. Total RNA was then isolated using TRIzol (GIBCO-BRL, Burlington, ONT) and was then used directly in an RT-PCR reaction with the following primers: forward 5'-AGACATAAGCTTGCAGCCATCATGGCGAACCTTGGC-3' and reverse 5'-GACATTCTCGAGCTACGATCCTCTCTGGTAATAGGC-3'. The forward primer includes a HindIII restriction site and the reverse primer includes both a XhoI restriction site and a TAG stop codon (to terminate translation following Ser^{230}). One ug of total RNA was added to the reaction mixture containing PCR buffer, 0.5 mmol/L dNTPs (Pharmacia), 50 pmol of each primer, 0.5 uL AMV-RT, and 0.5 uL of Taq polymerase (both enzymes from GIBCO-BRL). Reactions were carried out in a thermocycler (Perkin Elmer Cetus Corp, Norwalk, CT) for 40 cycles of 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. The size of the PCR product was verified by electrophoresis in a 1% TAE agarose gel, the fragment excised and gel purified, digested with HindIII and XhoI and ligated into pCDM8.

PrP-Ig

The generation of a 721 bp fragment encoding the human PrP ORF up to and including Ser^{230} was completed in a similar manner as described for PrPΔ. The forward primer used for amplification was

identical to that used for PrPΔ. The human PrP-Ig reverse primer, 5'-GAGATTCTCGAGTCTCACCTGACGATCCTCTCTGGTAATA-3', contains a XhoI restriction site followed by the splice donor sequence (TCTCACC) to insure correct mRNA processing and splicing of the human PrP ORF to the hinge sequence of human IgG1. After amplification, the PCR product was digested with HindIII and XhoI and ligated into pCDGI (plasmid kind gift from Dr. Marilyn Kehry, Boehringer Ingelheim, Ridgefield, CT).

SEAP/CMV

The fragment containing the entire coding sequence of SEAP, including an N-terminal signal peptide, was excised from pSEAP-Control Reporter Vector (Clontech, Palo Alto, CA). The plasmid was digested with ClaI, blunted with T4 polymerase (according to Maniatis) and then digested with HindIII. The SEAP fragment was gel purified and subcloned into HindIII/EcoRV digested pCDNAIAmp (Invitrogen, Carlsbad, CA).

PrP-AP

For the PrP-AP fusion protein, a 707bp PrP gene fragment was generated by PCR from mouse brain cDNA, which encoded the entire open reading frame from the initiating methionine (thus containing the PrP leader sequence) to Arg^{229} at the beginning of the GPI anchor attachment signal sequence (thus obviating the attachment of the GPI anchor). The forward primer was identical to the one used for the first two PrP constructs, since murine and human sequences are virtually identical at the 5' end of the PrP ORF. The murine PrP-AP reverse primer, 5'-GAGATTGGATCCTCTTCTCCCGTCGTAATAG-3',

contains a BamHI site which generates a Gly-Ser-Ser-Gly linker between PrP and AP when ligated to the BglII site of the AP-tag-2 expression vector (kind gift of Dr. J.G. Flanagan, Harvard University) which contains the AP gene downstream from the cassette insert site.

Cell Culture & Transfection

Cell lines were maintained in DMEM (GIBCO BRL) supplemented with 10% FBS, 2.5 mg/ml penicillin, 2.5 mg/ml streptomycin, and 2mM glutamine (hereafter referred to as complete DMEM).

COS-7 cells were transiently transfected using either DEAE-Dextran or Superfect (Qiagen, Santa Clarita CA). Cells were allowed to recover for 24 hours post-transfection in complete DMEM (as above) and were then trypsinized, washed twice in complete DMEM (to inactivate trypsin) and reseeded in T75 flasks in either complete DMEM or serum-free OPTI-MEM (GIBCO-BRL). The cells were then grown for 5 to 7 more days and the conditioned media was then collected, centrifuged at 12000 x g and kept at 4°C with 20 mM Hepes pH 7.0 and 0.1% sodium azide.

Binding assays

Use of PrP-AP in binding assays for detection of PrP binding proteins was done essentially as described for other AP fusion proteins (Cheng and Flanagan, 1994; Flanagan and Leder, 1990). Briefly, target cells were plated in 60mm dishes or 6 well plates (Nunc, Naperville, IL) and allowed to grow to confluency. Cells were rinsed with cold HBHA (Hank's balanced salt solution with 0.5 mg/ml BSA, 0.1% sodium azide, and 20 mM HEPES [pH 7.0]) and then overlayed with either SEAP or PrP-AP containing conditioned media,

normalized to contain equal amounts of AP activity. Cells were incubated at room temperature for 90 minutes in the dark. Following incubation, cells were rinsed six times with cold HBHA buffer and then lysed in cold Triton X-100 lysis buffer. Lysate was harvested, vortexed well, and allowed to extract on ice for a further 15 minutes. Lysate was then heat inactivated at 65°C for 10 minutes, centrifuged at 12000xg for 5 minutes, and assayed for AP activity using the BCIP solution reagent (Kirkegaard&Perry Labs, Gaithersburg, MD). Optical density measurements were done using an ELISA plate reader at 620 nm.

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Figures

Figure 3.1: Characteristic features of the normal cellular isoform of the prion protein

PrP has an N-terminal signal peptide (SP), followed by a glycine-proline rich octapeptide repeat region (G-P repeat), an intrachain disulfide bond, two sites of N-linked glycosylation and a C-terminal hydrophobic sequence that directs the attachment of the GPI anchor. The complete protein sequence is 253 amino acids in human and 254 amino acids in mouse. The PrPΔ construct comprises the entire sequence of human PrP until and including the Ser^{230} codon, thereby deleting the GPI anchor attachment sequence to generate a secreted form of PrP^{c}.

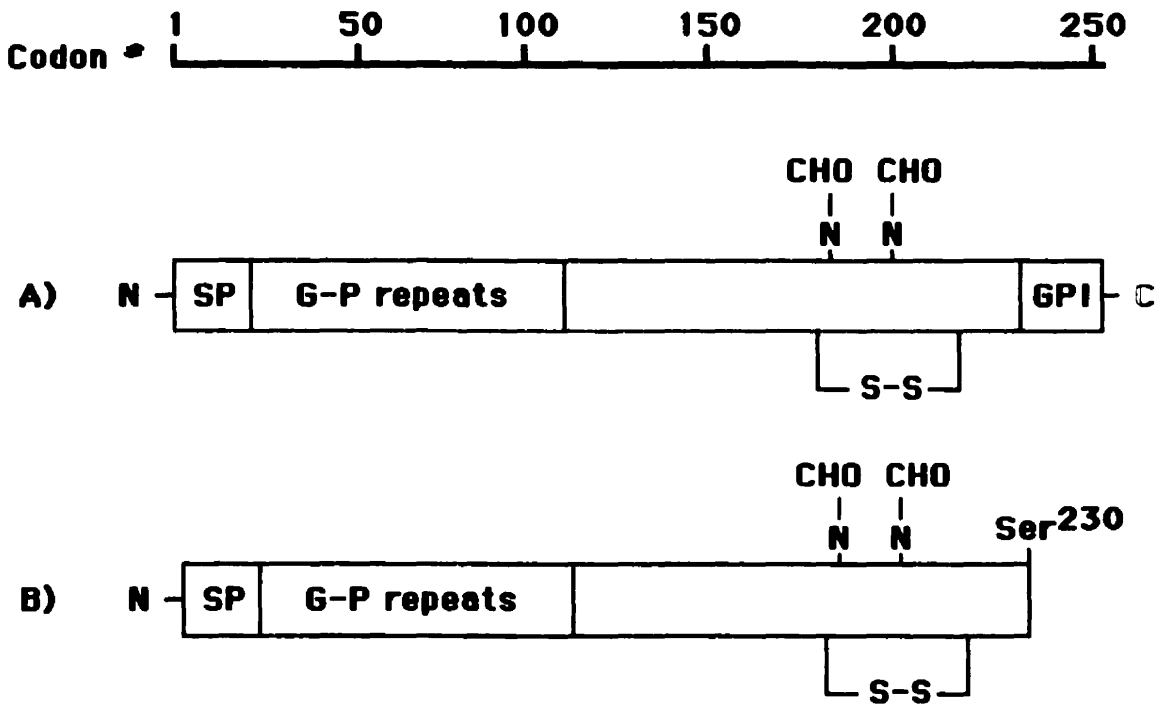

Codon
1
50
100
150
200
250
CHO
CHO
N
N
A)
N
SP
G-P repeats
GPI
C
S-S
CHO
CHO
N
N
Ser230
B)
N
SP
G-P repeats
S-S

Figure 3.2: Design of the PrP-Ig expression vector

A fragment of human PrP sequence up to and including the Ser^{230} codon was amplified by polymerase chain reaction using specific primers that contained additional restriction sites for cloning into the pCDG1 vector. This vector contained the genomic hinge, CH2 and CH3 sequences of the human IgG1 heavy chain. The PrP reverse primer sequence therefore also included a splice donor site for proper mRNA processing.

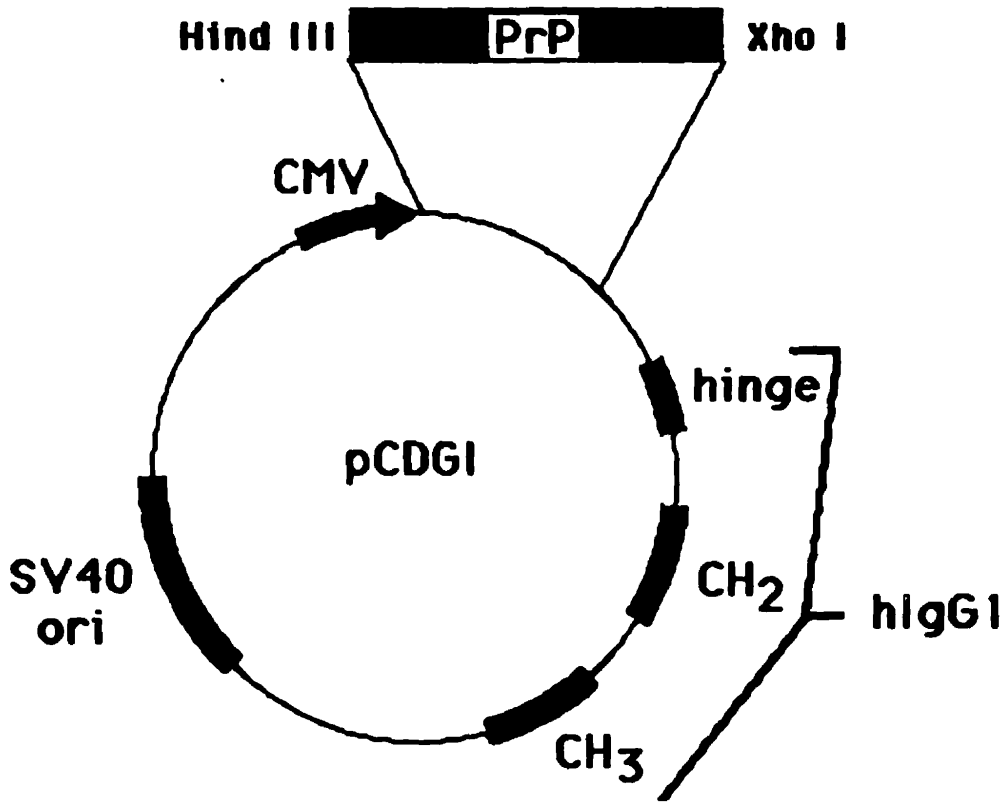

Hind III
PrP
Xho I
CMV
hinge
pCDG1
SV40
ori
CH2
hIgG1
CH3

Figure 3.3: Production of the PrP-Ig recombinant soluble fusion protein

A) COS-7 cells were transfected with either the control pCDM8 vector (lane 1) or the PrP-Ig expression vector (lane 2) and metabolically labeled with ^{35}S methionine. Supernatants were harvested and PrP-Ig protein precipitated with immobilized protein A. Samples were separated by SDS-PAGE and autoradiographed. PrP-Ig monomers are approximately 65-67 kDa.

B) PrP-Ig protein is predicted to be secreted as a dimer due to the disulfide linkage of the Ig chains. For verification, PrP-Ig samples were prepared as above and either boiled in SDS-PAGE sample buffer (which releases PrP-Ig from protein A and dissociates the dimers, lane 1) or snap frozen in SDS-PAGE sample buffer (which releases PrP-Ig from protein A while preserving the dimers, lane 2). PrP-Ig monomers migrate at approximately 65-67 kDa while PrP-Ig dimers migrate at 130 kDa.

C) PrP-Ig protein is degraded when produced in serum-free medium. PrP-Ig protein was precipitated with immobilized protein A from supernatants of control (lane 1) or PrP-Ig (lane 2) transfected COS-7 cells. Samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted with ^{125}I labeled goat anti-mouse Ig (cross-reactive with human Ig). Lane 2 shows a product migrating at approximately 35 kDa, indicating that most of the PrP portion of the protein is missing.

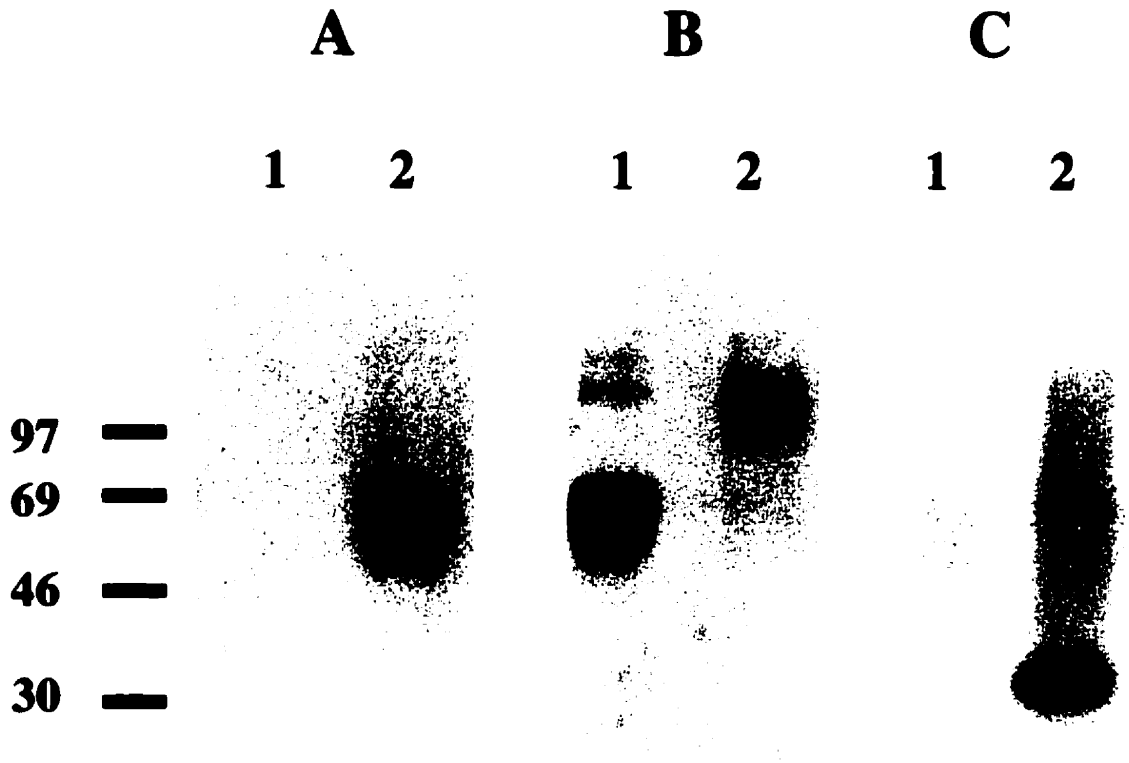
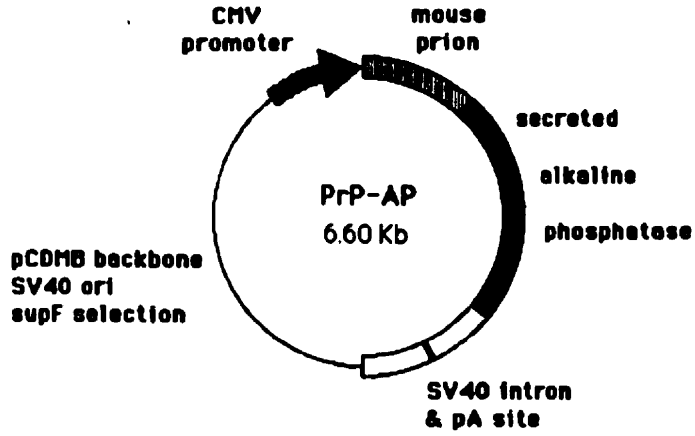

A
B
C
1
2
1
2
1
2
97
69
46
30

Figure 3.4: Design of the PrP-AP and SEAP expression vectors

The PrP-AP expression plasmid was constructed by cloning of a PCR fragment encoding murine PrP up to and including the Arg^{229} codon into the APtag-1 vector. This generated an in-frame fusion with the secreted alkaline phosphatase sequence included in the vector. The pCMV/SEAP control expression plasmid consists of the secreted alkaline phosphatase sequence preceded by its own signal peptide leader sequence.


CMV
promoter
mouse
prion
secreted
alkaline
phosphatase
PrP-AP
6.60 Kb
pCDMB backbone
SV40 ori
supF selection
SV40 intron
& pA site

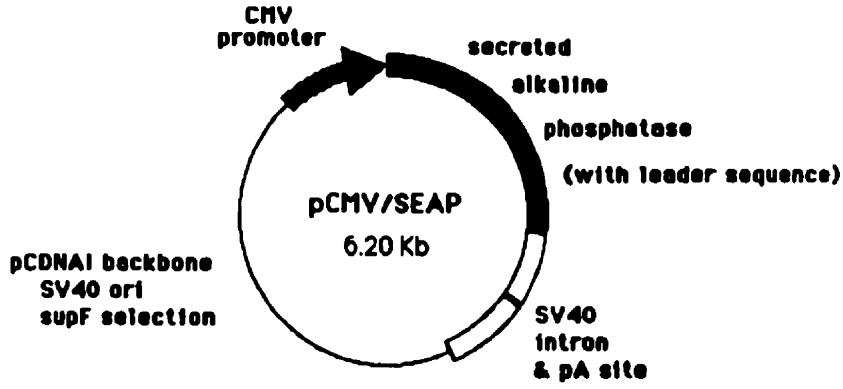

CMV
promoter
secreted
alkaline
phosphatase
(with leader sequence)
pCMV/SEAP
6.20 Kb
pCDNAI backbone
SV40 ori
supF selection
SV40
intron
& pA site

Figure 3.5: Expression of the PrP-AP and SEAP proteins

COS-7 cells were transfected with either the CMV/SEAP or the PrP-AP vector and metabolically labeled with ^{35}S methionine. Supernatants were harvested and immunoprecipitated with either an anti-alkaline phosphatase monoclonal antibody (lanes 1 and 3) or an anti-PrP polyclonal antibody (lanes 2 and 4). The anti-PrP polyclonal was raised against the N-terminal peptide 23-40 and is of low titer. Samples were separated by SDS-PAGE and autoradiographed.

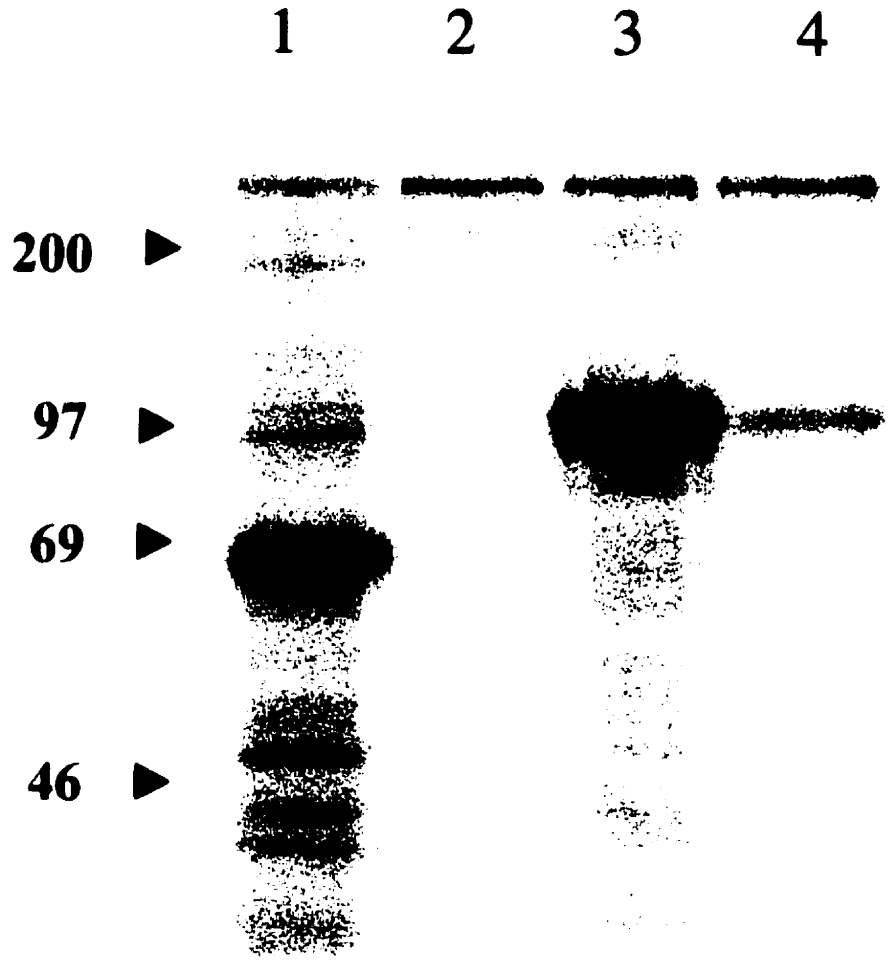
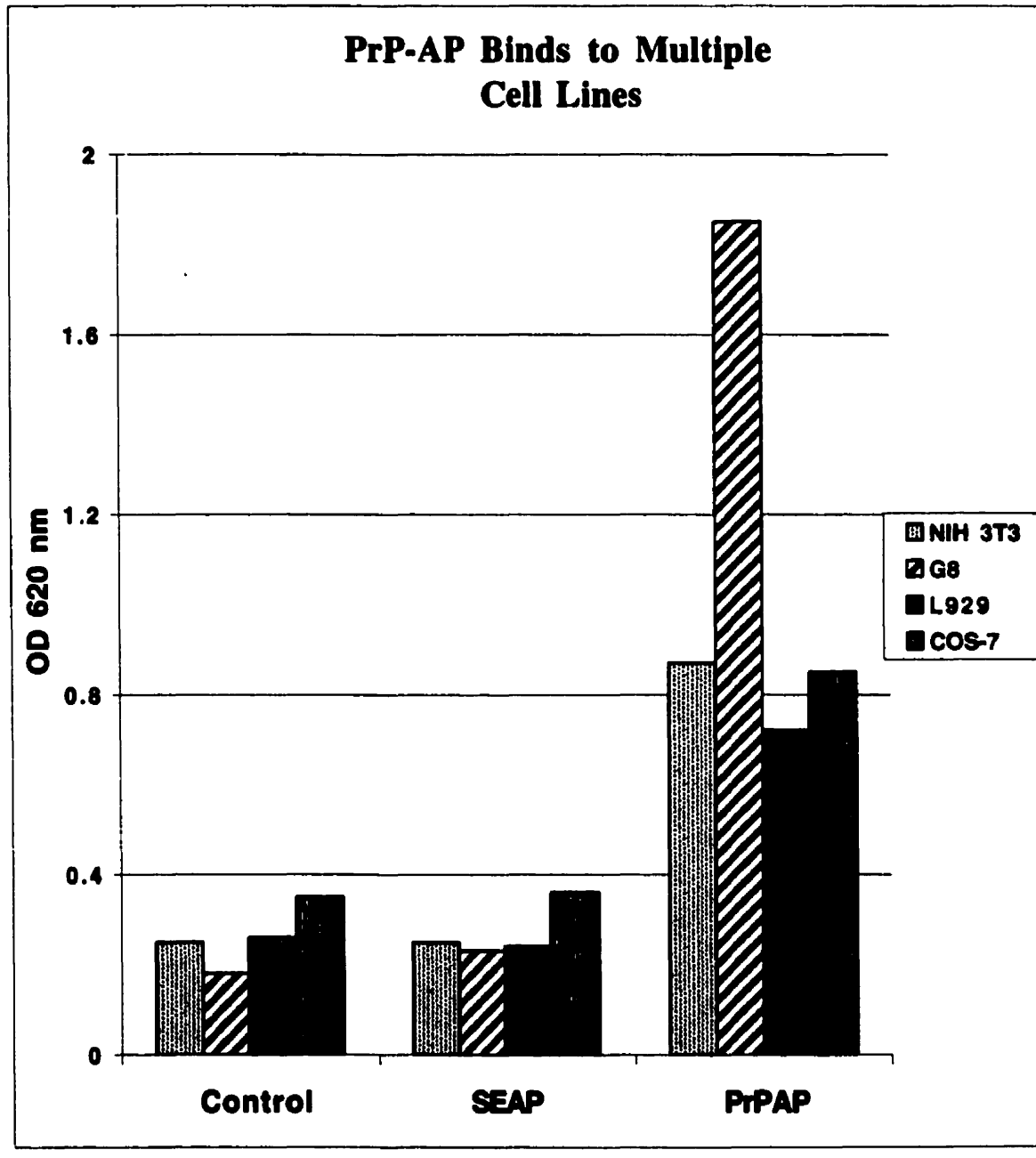

1
2
3
4
200
97
69
46

Figure 3.6: PrP-AP binding activity of several cell lines

Cells were incubated with control, SEAP or PrP-AP supernatants, washed, lysed, and assayed colorimetrically for bound AP activity. Control represents conditioned media from mock transfected cells containing no detectable AP activity. Equimolar amounts of SEAP reveals no binding relative to control, whereas PrP-AP exhibits binding to all cell lines shown.


PrP-AP Binds to Multiple Cell Lines
OD 620 nm
2
1.6
1.2
0.8
0.4
0
Control
SEAP
PrPAP
NIH 3T3
G8
L929
COS-7

Following the generation of three different soluble prion constructs, we successfully used the prion-alkaline phosphatase fusion protein (PrP-AP) to confirm the existence of a cell surface binding activity specific for PrP. This chapter describes the further use of PrP-AP to study the interaction with this potential receptor in greater detail.

Chapter 4

Characterization of a Cell Surface Receptor for the Prion Protein [1]

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Contributions of additional authors

Dr. Bob Chalifour was responsible for the design and execution of the purification of PrP-AP. All subsequent experiments involving the use of this purified reagent were performed by the author.

Summary

The normal isoform of the prion protein (PrP^C) is a small cell surface glycoprotein that is expressed in a wide variety of tissues. The cell surface localization of this protein suggests that it may interact with other molecules on the same or adjacent cells. We have designed and expressed a secreted, alkaline phosphatase tagged form of the prion protein (PrP-AP) to assay for the existence of cell surface prion binding proteins. We have determined that PrP-AP binds to the surface of a multitude of cell lines in a specific, saturable, high-affinity fashion, and that this interaction depends on a protein component. We demonstrate that this interaction is dependent on the N-terminal region of the protein and can be blocked by corresponding N-terminal peptides. Copper is shown to play a role as a structural co-factor, most probably by conferring a favourable binding conformation to the N-terminal domain. These data suggest the existence of a widely expressed high-affinity receptor for the prion protein.

Introduction

Prions are the infectious agents involved in a class of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSE's) or prion diseases. The major, if not the only, component of this agent is PrP^{Sc}, a protease resistant form of the normal cell surface protein PrP^{c}. The two forms of this protein differ dramatically in secondary structure and biochemical properties (Caughey et al., 1991; Safar et al., 1993a; Safar et al., 1993b). It is hypothesized that contact between the two forms is necessary for conversion of PrP^{c} to PrP^{Sc} by a post-translational process, which probably occurs in endosomes (Borchelt et al., 1992). Mutations in the PrP^{c} protein sequence that are linked with disease are postulated to facilitate a process of spontaneous conversion.

Although the role of PrP^{Sc} in disease has been highly characterized, little is known of the function of its normal counterpart, PrP^{c}. This small cell-surface sialoglycoprotein is anchored by a glycosylphosphatidyl-inositol (GPI) linkage to the outer leaflet of the plasma membrane. It is predominantly expressed on neurons and glia in the central nervous system but also in a wide variety of peripheral tissues (Bendheim et al., 1992; Dodelet and Cashman, 1998; Moser et al., 1995). The expression of PrP^{c} begins early in development, it is present in all the vertebrates examined to date and portions of the PrP gene sequence are highly conserved throughout evolution, suggesting a crucial role for the protein

(Manson et al., 1992; Westaway and Prusiner, 1986). Strikingly, mice homozygous for a null mutation of the PrP gene do not demonstrate any developmental abnormalities, but present more subtle phenotypes such as defective $GABA_A$ receptor-mediated fast inhibition and impaired long-term potentiation, altered circadian rhythms, and loss of cerebellar Purkinje cells with advanced age (Bueler et al., 1992; Collinge et al., 1994; Sakaguchi et al., 1996; Tobler et al., 1996). However, null mice are resistant to infection with PrP^{Sc}, showing that the expression of PrP^{c} is indispensible for the disease process to occur (Bueler et al., 1993).

Although the normal function of PrP^{c} is unclear, the localization of the protein at the cell surface argues for a role in cell adhesion, signaling, or uptake of soluble extracellular molecules. It is therefore likely that PrPc interacts with one or more ligands at the cell surface. Recently, two groups have attempted to identify the target ligand(s) for PrP^{c}. Martins et al have used a complementary hydropathy strategy to generate an antibody for a PrP^{c} 'receptor' and have identified a 66 kDa protein present on neurons that binds to PrP^{c} (Martins et al., 1997). The nature of this protein is unknown at present. A two-hybrid screen was used by Rieger et al to identify PrP^{c} interacting proteins, one of which was the 37 kDa laminin receptor precursor (Rieger et al., 1997). The difference in size of these two candidate proteins indicates that they are probably not the same, and the lack of physiological data makes it impossible to determine whether either one is the *bona fide* receptor for PrP^{c}.

However, it is clear that PrP^c can interact with other cell surface proteins and that some or all of these interactions may be relevant for the normal function of PrP^c.

To further these studies, we have constructed a soluble form of the prion protein fused to human placental alkaline phosphatase to determine the nature of the interaction between PrP^c and its putative ligand(s). We report here that this recombinant soluble form of PrP^c binds to a variety of mammalian cell types in a saturable and specific manner. This binding is dependent on an intact N-terminus of the prion protein, and can be specifically blocked by N-terminal peptides. Copper has been reported to bind to an N-terminal region of PrP^c defined by amino acids 23-98 and we demonstrate that copper is a necessary co-factor for the binding activity of PrP^c to the cell surface. Additionally, we demonstrate that the N-terminus of native cell surface prion protein is one of only two regions of the protein that are exposed and thus likely targets for interaction with putative ligands. Our results suggest that both the N-terminus of the prion protein and copper contribute to normal PrP^c structure and its ability to interact with one or more cell surface ligands.

Results

Expression of PrP fusion proteins

In order to search for potential ligands for the prion protein, several secreted recombinant forms of the protein were constructed. A

truncated form lacking the C-terminal hydrophobic signal sequence, and thus the GPI anchor, was generated and successfully expressed. However, the protein proved too difficult to produce in sufficient quantity and was therefore not a feasible tool for binding studies. A fusion with the constant portion of human IgG1 heavy chain was then generated and proved to be stable and readily produced by transient transfection. However, no binding activity was detectable with this fusion protein (reviewed in the previous chapter). Finally, a fusion with human placental alkaline phosphatase (PrP-AP) was generated and successfully expressed. Figure 4.1 shows that PrP-AP is a secreted protein, produced as a single major polypeptide with the expected molecular mass of approximately 97 kDa, relative to 67 kDa for secreted alkaline phosphatase (SEAP) alone. This fusion protein has the advantage of carrying a tag that can be bound by commercial monoclonal antibodies and that has enzyme activity, which can be quantitated by simple colorimetric assays without need for purification or labelling.

Binding of PrP-AP is specific and of high affinity

To investigate the presence of potential prion protein ligands on cell surfaces, a variety of cell lines were incubated with PrP-AP conditioned media, washed, lysed, and the lysates tested colorimetrically for bound AP activity. All of the cell lines assayed exhibited binding to PrP-AP, albeit to varying degrees (Figure 4.2A). This wide distribution of binding activity is not surprising given the nearly ubiquitous expression patterns of PrP^c itself (Bendheim et al., 1992). Scatchard analysis of the binding to G8 cells indicated that it

is saturable and produced Kd values of approximately 25 x 10^{-9} M, with 1.5 x 10^5 binding sites per cell (Figure 4.2B), indicating a high-affinity interaction within the range typical for receptor-ligand binding. The Kd of approximately 25 x 10^{-9} M is ten-fold lower (meaning higher affinity) than that reported for a bacterially produced fusion protein of PrP^c (Shyng et al., 1995). This may be due to more adequate folding or post-translational modifications such as glycosylation during production in a mammalian versus a bacterial system. Additionally, the human placental alkaline phosphatase fusion partner has been reported to dimerize non-covalently, which could increase the measured affinity by presenting a divalent form of PrP^c to the cell surface.

To determine if PrP^c was binding to a proteinaceous receptor, COS-7 cells were pretreated with trypsin before carrying out a binding assay. Figure 4.3 shows that binding to COS-7 cells is ablated by the trypsin pretreatment, implicating a protein component.

The N-terminus of PrP functions as a binding domain

We observed batch to batch variations in binding profiles for PrP-AP containing supernatants. Although the supernatants exhibited similar amounts of AP activity, immunoprecipitation of the fusion protein from these various batches revealed small variations in molecular weight (mw). A strong correlation was then established between a lower mw product and lack of binding activity (Figure 4.4).

Since the AP activity was intact and N-terminal truncated forms of the prion protein have been reported (Harris et al., 1993), we

hypothesized that the observable shift in molecular weight was due to a small amount of degradation at the N-terminus of the fusion protein. Immunoprecipitation with an N-terminus specific Ab revealed that, indeed, the N-terminus was lacking in the lower mw fusion protein (Figure 4.5). These results suggest that the N-terminus of the prion protein is involved in the binding activity observed. This involvement may be indirect as this region of the protein could contribute to the proper conformation or overall stability of the structure.

NMR studies of purified, bacterially produced forms of PrP^{c}, have shown the N-terminal polypeptide segment to be flexibly disordered and structured as a random coil (Donne et al., 1997; Hornemann et al., 1997; Riek et al., 1997). This flexibility, while postulated to ease interaction with PrP^{Sc}, may thus also contribute to the binding of a heterologous receptor. We decided to examine the gross topology of normal cell surface PrP^{c} by flow cytometric analysis of a T lymphocyte cell line using antibodies directed against several different peptide regions of the protein. This revealed that the N-terminus is one of the few surface-accessible epitopes of the prion protein in its native conformation at the cell surface (Figure 4.6). This availibility of the N-terminus for contact would argue for a more direct involvement as a binding domain for PrP^{c}.

To further the analysis of the binding capacity of the N-terminus of PrP^{c}, peptide blocking experiments were carried out. Peptides

corresponding to the first 5 or 11 amino acids of the mature form of PrP^c (beginning after the signal peptide) were resuspended in PrP-AP containing conditioned media, which was then used in a binding assay. Figure 4.7 demonstrates that the 11-mer peptide is capable of blocking binding of PrP-AP to the surface of both COS-7 and G8 cells, while the 5-mer peptide had no effect (data not shown). The 11-mer peptide most probably acts in a direct, competitive manner, binding to the active site of the receptor. This confirms the involvment of the N-terminus in binding of PrP^c to its receptor.

Copper as a structural co-factor for PrP^c

In an attempt to generate a purified fraction of PrP-AP, we developed a purification protocol based on phosphonate gel chromatography. High concentrations of the chelating agent EDTA (0.01M) were necessary for elution. The resulting fractions were then dialyzed against a magnesium and zinc ion containing buffer to restore these ions for proper AP function. However, when these fractions were tested in a binding assay, they proved to be very weak, although their AP activity content was high. It has been reported that the octapeptide repeat region in the N-terminal half of the prion protein binds to copper, and the presence of this ion may serve to coordinate proper structure of the protein (Hornshaw et al., 1995; Hornshaw et al., 1995; Stockel et al., 1998). We investigated the possibility that copper may have been chelated away from PrP-AP during the purification procedure and that this metal may be a

necessary co-factor for PrP-AP binding activity. Addition of 1 mM copper to non-active eluted fractions of PrP-AP restored the binding

activity to expected levels. However, the addition of copper did not improve the binding of a purified PrP-AP fraction with N-terminal degradation (Figure 4.8). This demonstrates that copper may aid in the proper folding and organization of an intact N-terminus and is a necessary and specific co-factor for the binding activity of PrP^c.

Discussion

In this paper we have described a cell surface binding activity for a prion protein-alkaline phosphatase fusion protein, representing a potential ligand for cellular PrP^c. We based our approach on the supposition that, as a protein with potential involvement in cell signalling and/or adhesion, PrP^c was likely to interact with one or more cell surface ligands that could be detected by a tagged recombinant form of the protein. Our data indicate that this binding activity is specific, saturable and of high affinity, and dependent upon an intact N-terminus of the PrP^c moiety of the fusion protein. Further analysis revealed that this binding activity could be competed with peptides corresponding to the N-terminal amino acids of the mature protein. We extended our observations to cell-surface PrP^c, where flow cytometry studies showed that the N-terminus, along with a more central epitope, are the only two regions of the protein, out of 10 domains studied, to be exposed and thus more likely to be available for interactions with potential ligands. Finally, we determined that copper, which has been shown to bind the N-terminal octarepeat region of the protein (Hornshaw et al., 1995; Hornshaw et al., 1995; Stockel et al., 1998), may indeed serve as a

structural cofactor in that it restored the binding activity of a purified fraction of PrP-AP.

Our findings strongly implicate the N-terminus of PrP^{c} as a crucial element of the protein which may be essential to its normal function. Analysis of this region of the protein has been somewhat neglected due to its apparent lack of involvement in prion diseases. The scrapie isoform, PrP^{Sc}, is known to have a protease resistant core, where approximately 60-70 N-terminal amino acids are lost upon protease treatment, without any observable alteration in infectivity. A truncated construct expressed in scrapie infected N2A neuroblastoma cells demonstrated that PrP^{c} devoid of 66 N-terminal amino acids can still give rise to PrP^{Sc}, although infectivity was not determined (Rogers et al., 1993). Prion knockout mice made transgenic for PrP lacking 26 or 49 amino-proximal amino acids were equally susceptible to disease induction as knockout mice transgenic for wild-type PrP, with similar accumulations of PrP^{Sc} (Fischer et al., 1996). The N-terminus of PrP^{c} thus seems to be dispensible for its conversion to PrP^{Sc} and its role in prion disease.

However, studies on the post-translational processing and endocytosis of chicken PrP (chPrP) point to a role for the N-terminal region in the normal function of PrP^{c} (Harris et al., 1996). It was observed that, in N2A neuroblastoma cells transfected with chPrP, over half of the chPrP molecules on the cell surface at steady state are cleaved within an N-terminal region of 24 amino acids that is identical in chicken and mouse PrP^{c}. Immunoblots of conditioned media from these cells revealed the presence of two fragments of 10

and 30 kDa corresponding to the cleaved region (Harris et al., 1993). Additionally, the N-terminus was reported to be important for proper localization of chPrP to clathrin-coated pits at the cell surface, and for its subsequent internalization (Shyng et al., 1995). These experiments have led Harris and colleagues, much as ourselves, to postulate the existence of a receptor that would interact preferentially, if not exclusively, with the N-terminus of PrP^c (Harris et al., 1996).

The existence of a cell surface binding activity for PrP^c is confirmed by our studies for PrP-AP. The lack of multiple saturation plateaus in the interaction suggest a single type of receptor. Although PrP^c has been shown to interact with various glycans and has the heparin binding sequence motif (XBBXBX, where B= basic amino acid and X= other amino acids, (Cardin and Weintraub, 1989)) at the N-terminus, abrogation of PrP-AP binding by pretreatment of the target cells with trypsin implicates a protein component. The two findings are not mutually exclusive, implying that the receptor for PrP^c may be a proteoglycan.

Regardless of the nature of the receptor, it is clear that the N-terminus of PrP^c is necessary for the interaction and the apparent protease sensitivity of this region raises the interesting possibility that an N-terminal fragment may act as a soluble ligand, once released from the PrP core, for activation of its cognate receptor or competition with cell-bound PrP^c.

Although recent structural studies of PrP^c have determined that the N-terminal polypeptide is a flexible random coil (Donne et al., 1997; Hornemann et al., 1997; Riek et al., 1997), our data suggest that copper may confer a preferential binding conformation to this highly conserved domain. Indeed, copper has been shown to be coordinated by two histidine residues and two glycine carbonyl oxygens in the ocatapeptide repeat region and may impart a more rigid and organized structure to the N-terminus than has been implied from NMR analysis (Stockel et al., 1998). The reduced copper content in brain tissue from PrP null mice led to the hypothesis that cell surface PrP^c may be a copper binding protein in vivo involved in the supply or regulation of copper ion (Brown et al., 1997). By extension of this hypothesis, we propose that binding of copper to PrP^c changes the conformation of, minimally, the N-terminus and thus permits interaction of PrP^c-Cu^{2+} with its receptor. If this interaction occurs in cis, that is on the same cell, then internalization of the complex follows, presumably for uptake of copper. Potentially, this interaction could also occur in trans, with the receptor and PrP^c on different cells, by the release of either the copper bound N-terminus by protease cleavage or of the PrP^c holoprotein by PIPLC.

Given the importance of copper for normal enzymatic function and cellular metabolism, one would assume that a receptor for a copper binding protein would have wide, if not ubiquitous, tissue distribution much like PrP^c itself. Our data support this contention, in that all cell types examined bind PrP-AP to some degree. Interestingly, the muscle cell lines G8 and C2C12 exhibit the highest

degree of binding , perhaps reflective of the level of receptor expression in vivo. High level expression of PrP transgenes in both wild type and PrP null backgrounds led to pronounced hind limb paresis due to an impressive mitochondrial myopathy (Fischer et al., 1996; Westaway et al., 1994). Notably, the distribution of pathology was much more restricted than the distribution of protein expression, suggesting that the disease effect was transduced by a different molecule -- perhaps a PrP^c binding protein -- which is highly expressed in the cells affected by the myopathy.

Our results indicate that prion protein may indeed have a cell surface receptor and that interaction with this receptor is dependent upon an intact and copper bound N-terminal region. It will be of great interest to understand the identity of this receptor and the purpose of the interaction, as it is becoming increasingly clear that the normal biological function of the prion protein has many implications for its role in disease mechanism.

Materials & Methods

Production of AP fusion proteins

CMV/SEAP and PrP-AP were constructed and produced as previously described (see chapter 3). For metabolic labelling and immunoprecipitation, 24 hours post-transfection, COS-7 cells were rinsed with methionine free DMEM (GIBCO, Burlington, Ontario, Canada) and incubated overnight with 100 uCi/ml of Tran^{35}S-Label (ICN, Montréal, Québec, Canada) in methionine free DMEM supplemented with 2% dialyzed FBS. Supernatants were harvested, centrifuged at 12000 x g and labelled AP fusion proteins were then immunoprecipitated with a monoclonal antibody to against human placental AP (Medix Biotech, San Carlos, CA).

Quantitative cell surface binding and peptide blocking

Quantitative cell surface binding assays were performed as previously described (see chapter 3). For Scatchard analyses, conditioned media was concentrated in an ultrafiltration cell (Amicon, Oakville, Ontario, Canada) and dilutions were made with HBHA (Hank's balanced salt solution with 0.5 mg/ml BSA, 0.1% sodium azide, and 20 mM HEPES pH 7.0). Cells were then washed four times with HBHA, lysed and assayed colorimetrically for bound AP activity.

For the peptide blocking experiments, peptides were resuspended at 1 mg/ml in PrP-AP conditioned media, and the binding assay was carried out as previously described.

Purification of PrP-AP

Phosphonate gel (L-histidyldiazobenzylphosphonic acid, Sigma, St-Louis, MO) chromatography was used for PrP-AP purification. Conditioned media were dialyzed against phosphonate binding buffer (pbb; 0.01 M MES pH6.0, 0.1M NaCl, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$) and loaded onto the phosphonate gel column. The loaded column was then washed successively with pbb containing 0.3 M NaCl, pbb containing 0.9 M NaCl, and 0.01 M MES pH 6.0, 0.3 M NaCl, 0.01 M EDTA, 0.05 PO_4. The column was then eluted with 0.01 M MES pH 6.0, 0.8 M NaCl, 0.01 M EDTA, 0.05 PO_4. The eluate was then dialyzed against 0.01 M Tris pH 8.0, 0.1 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$ to eliminate free PO_4 and restore the necessary divalent cations for AP function.

Flow cytometry

Rabbit polyclonal anti-prion peptide antibodies were a kind gift of Drs P. Piccardo and B. Ghetti, Indiana State University. The peptides used were derived from the human prion sequence (Genbank accession number M13899) and included the following: 23-40, 58-71, 90-102, 95-108, 109-112, 151-165, 168-178, 178-188, 182-196, and 220-231.

For immunofluorescence staining, CEM human T lymphoma cells were first incubated on ice for 30 minutes in PBS supplemented with 10% normal goat serum to block non-specific binding. Cells were then stained with the polyclonal antibody panel followed by a goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate (Jackson Immunoresearch, West Grove, PA), each for 30 minutes on ice.

Samples were analyzed on a FACSCAN flow cytometer using LYSYS II software (Becton Dickinson, Saint-Laurent, Québec, Canada).

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Figures

Figure 4.1: Expression of SEAP and PrP-AP

COS-7 cells were transfected with either unfused SEAP or PrP-AP fusion protein expression plasmids. Cells were matabolically labeled with ^{35}S-methionine for 16-18 hours and the supernatants harvested and immunoprecipitated with a monoclonal antibody against human placental AP. Samples were separated on a 10% polyacrylamide gel and imaged with a Phosphorimager.

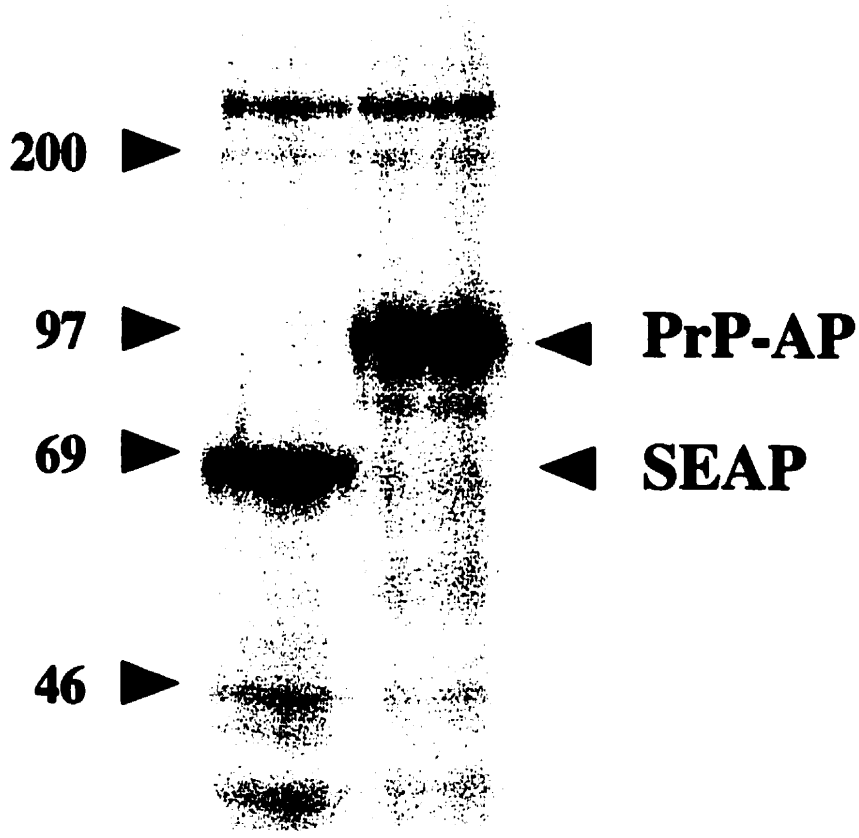

200
97
69
46
PrP-AP
SEAP

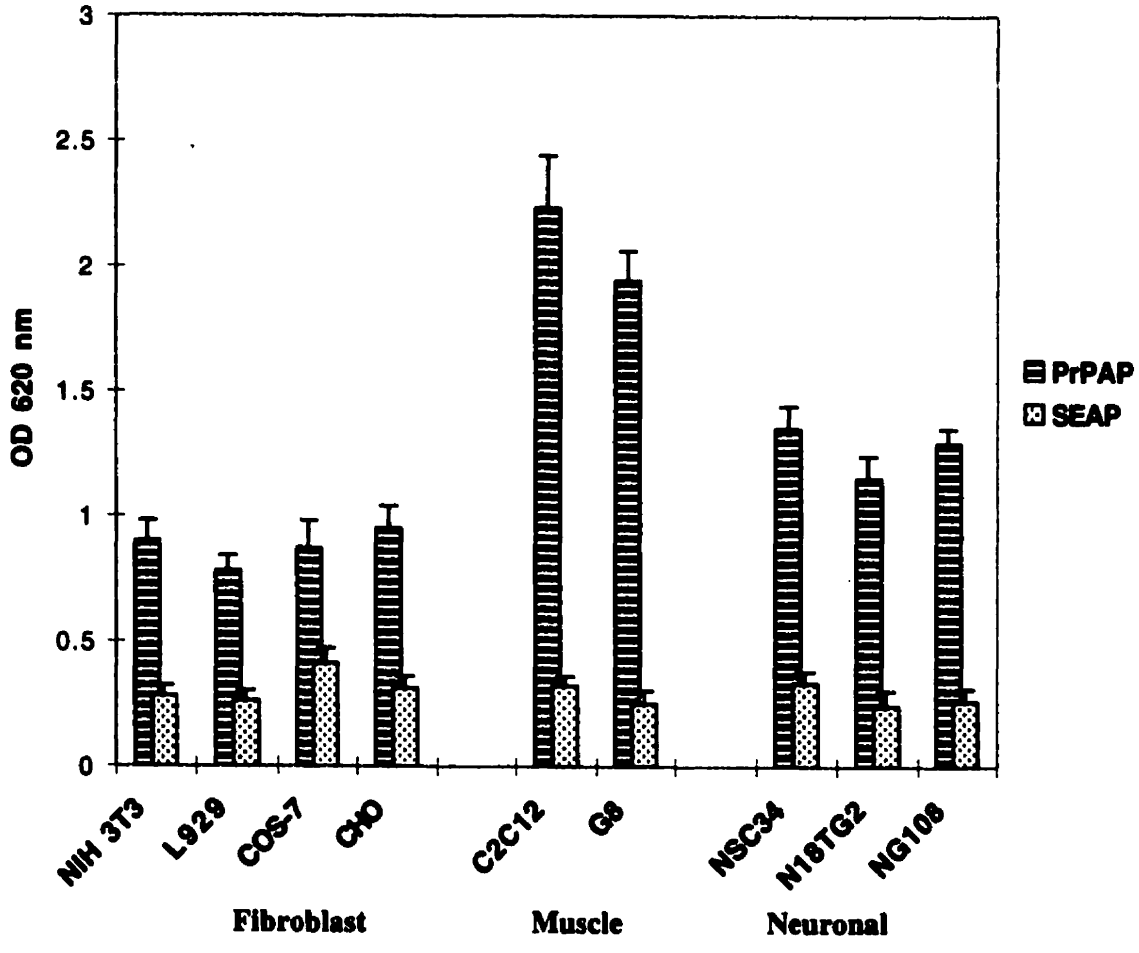
Figure 4.2A: PrP-AP binds to a wide spectrum of cell lines

Cell lines were treated with either SEAP or PrP-AP containing supernatants, washed, lysed, and assayed colorimetrically for bound AP activity. Each column shows the mean and range of values for two determinations.

2B: Scatchard analysis of PrP-AP binding

G8 cells were incubated with the indicated concentrations of PrP-AP, washed, lysed and assayed colorimetrically for bound AP activity. *Inset* Scatchard plot of the specific binding data, giving a calculated Kd of 25 nM with 1.5 x 10^5 sites per cell.

PrPAP Binds to Multiple Cell Lines



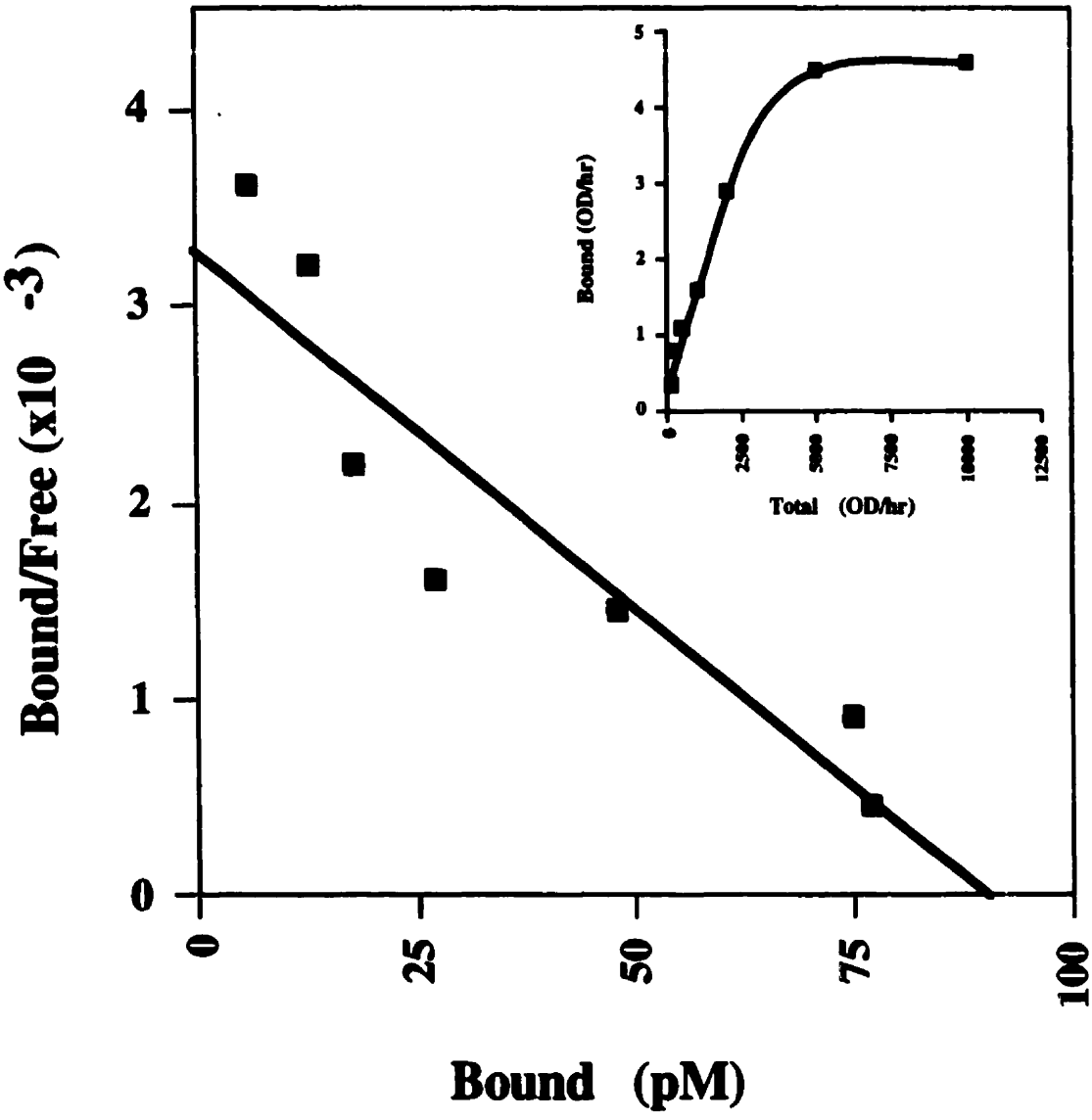

Bound/Free (x10 -3)
0
1
2
3
4
0
25
50
75
100
Bound (pM)
Bound (OD/hr)
Total (OD/hr)

Figure 4.3: Trypsin pre-treatment of COS-7 cells ablates PrP-AP binding

COS-7 cells were resuspended by titration and treated or not with a 0.25% trypsin solution for 15 minutes at 37°C. Cells were then washed twice with serum containing medium to inactivate trypsin and assayed for PrP-AP binding as described.

Trypsin Treatment of COS-7 Cells Ablates PrP-AP Binding

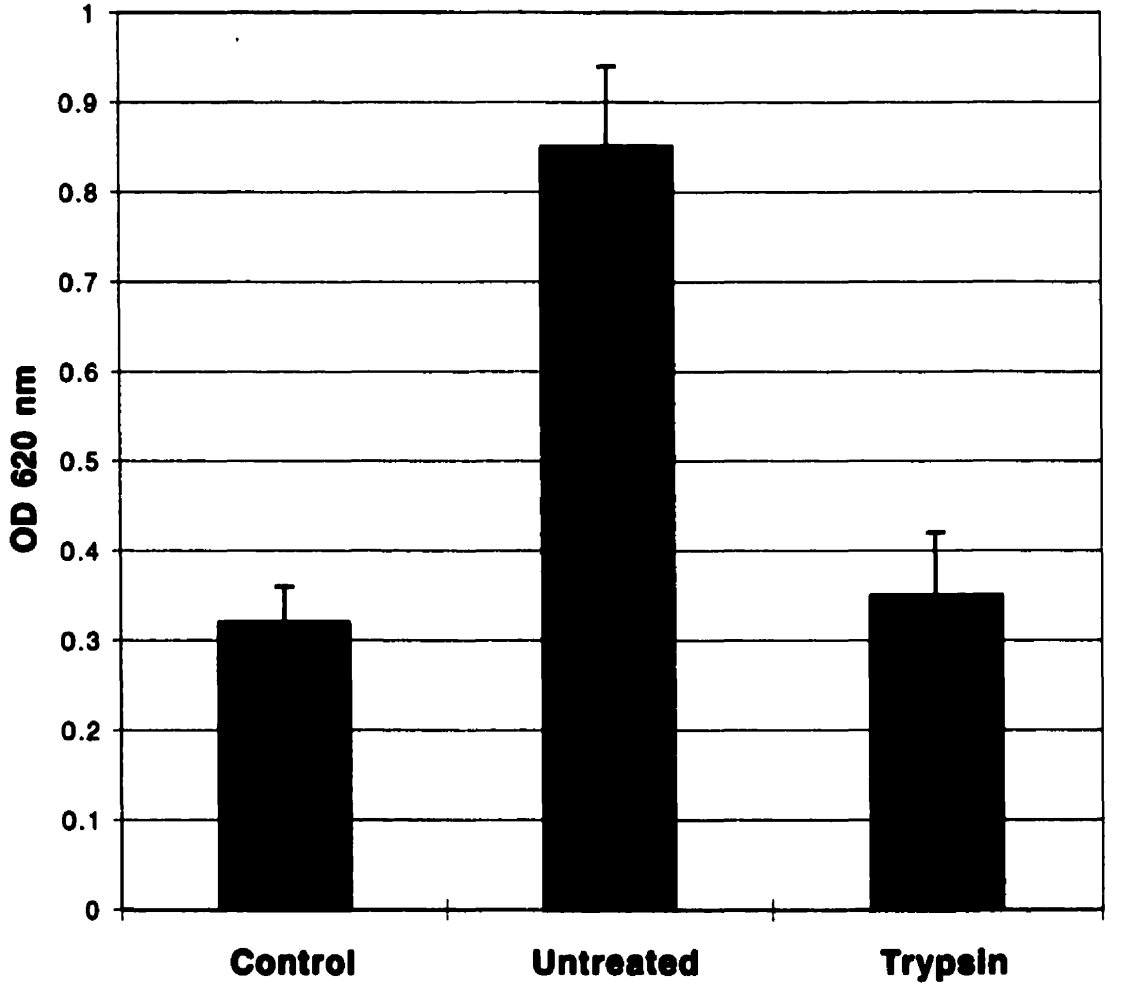


Figure 4.4: Truncated forms of PrP-AP show loss of binding activity

PrP-AP was immunoprecipitated from aliquots of two different batches of supernatant using an anti-AP monoclonal antibody. Samples were separated on a 10% polyacrylamide gel and stained with Coomassie blue. G8 cells were then assayed for PrP-AP binding using the two different supernatants. Loss of binding activity is observed from the supernatant containing the lower molecular weight form of PrP-AP. SEAP (background) binding is shown by dashed line.

PrPAP Binding Profiles: Sensitivity to Proteolysis

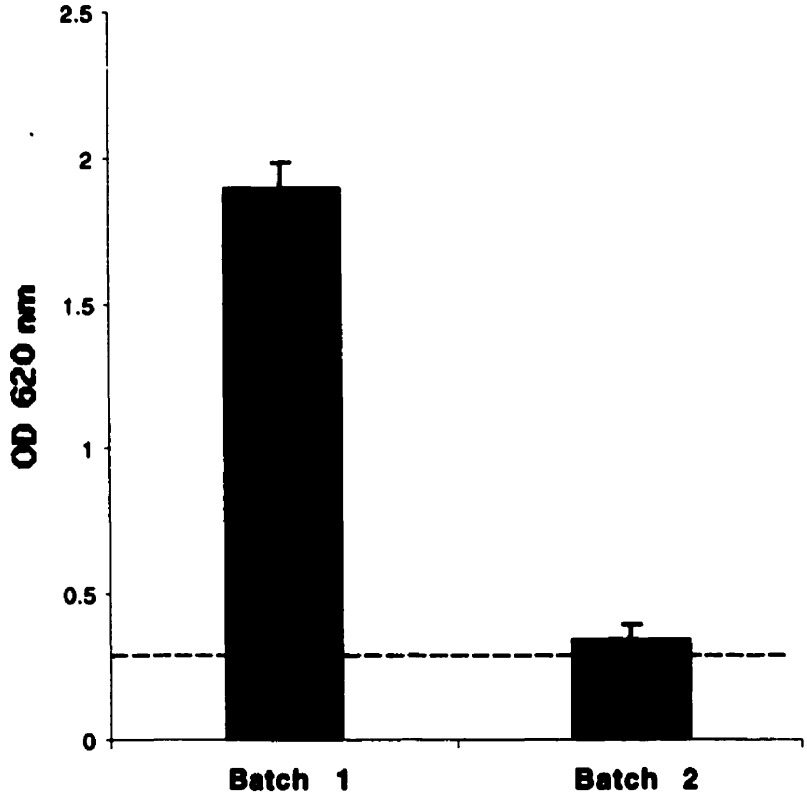


Figure 4.5: Truncation of N-terminus in lower molecular weight PrP-AP

Supernatants described in Figure 4 were immunoprecipitated with either an anti-AP monoclonal antibody (lanes 1 and 3) or a polyclonal sera directed against N-terminal amino acids 23-40 of PrP (lanes 2 and 4). Samples were separated on a 10% polyacrylamide gel and stained with Coomassie blue. The truncated form of PrP-AP cannot be immunoprecipitated with the polyclonal sera, indicating that the N-terminus of the protein is absent.

1 2 3 4

Figure 4.6: Surface topology of cell surface PrP^C

CEM T lymphoma cells were stained with a panel of anti-PrP peptide polyclonal sera and analyzed by flow cytometry. An Emini surface probability plot (top) is compared to surface epitopes as determined by flow cytometry (bottom). Positive or negative staining is denoted by +/- for the corresponding peptide. Amino acid positions are shown in the center.

Prion Protein Surface Epitopes

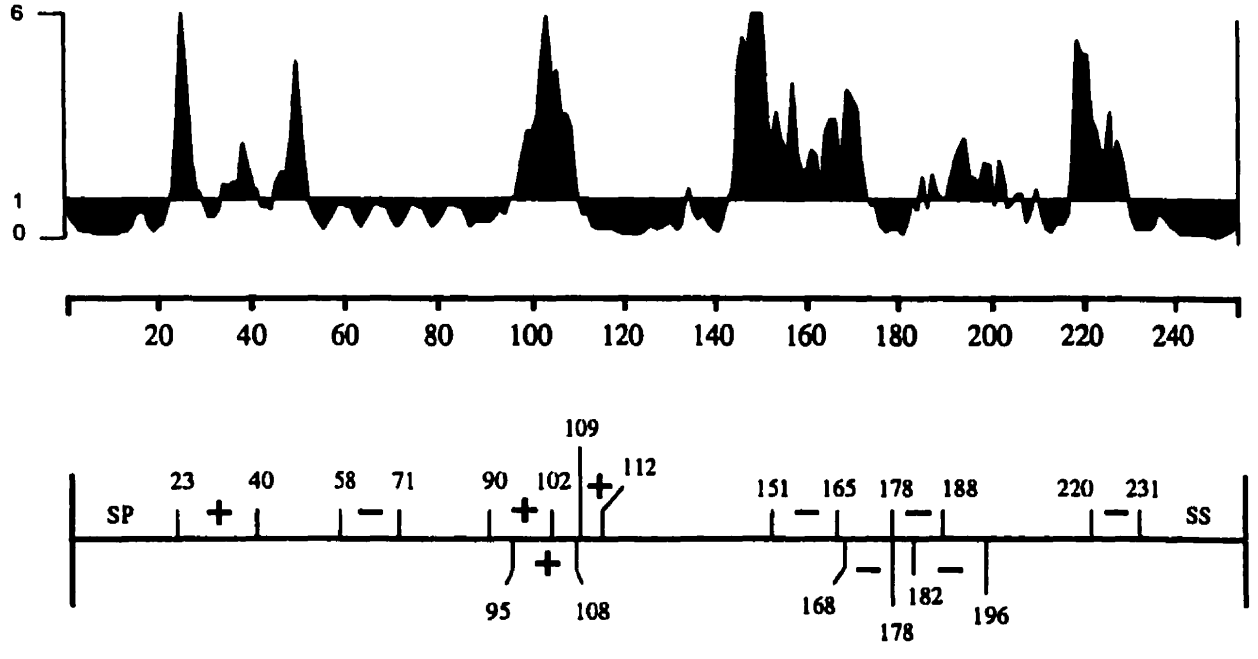


Figure 4.7: A prion N-terminal peptide blocks PrP-AP binding

An 11-mer peptide corresponding to amino acids 23-33 of PrP was added to PrP-AP containing supernatant to a final concentration of 1 mg/ml. COS-7 and G8 cells were then treated with SEAP, PrP-AP or PrP-AP + peptide supernatants and assayed for bound AP activity.

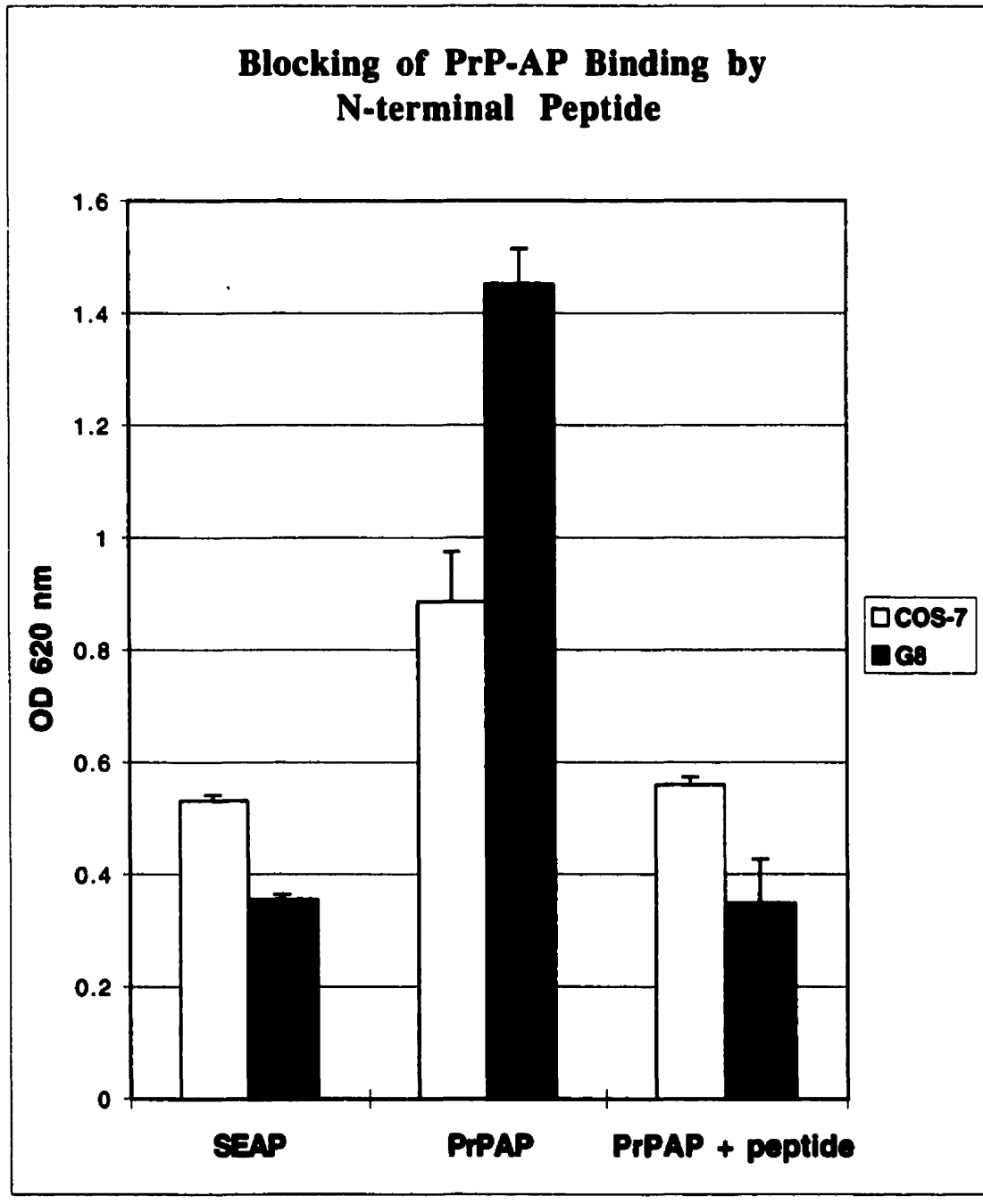
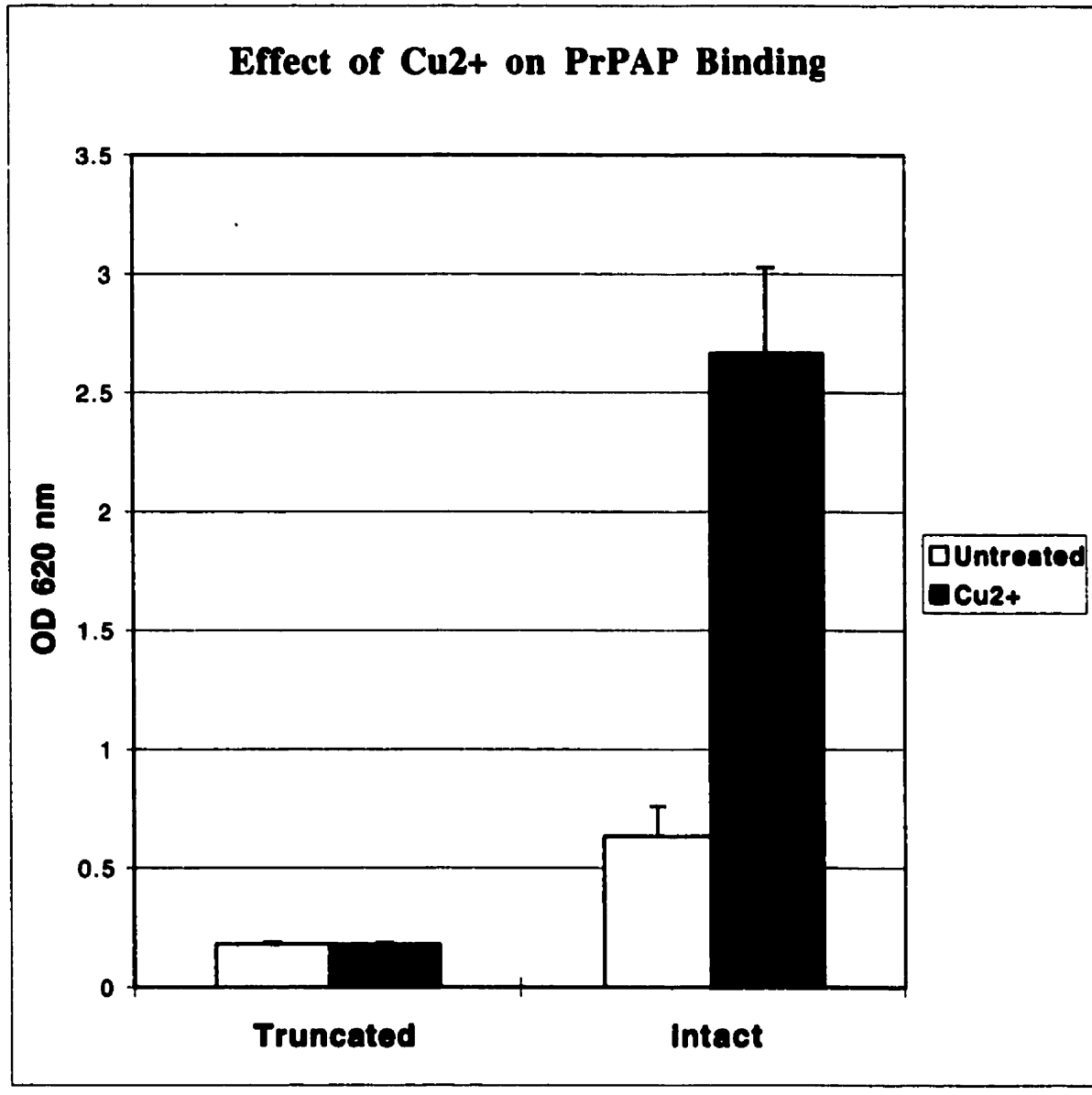

Blocking of PrP-AP Binding by
N-terminal Peptide
OD 620 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SEAP
PrPAP
PrPAP + peptide
COS-7
G8

Figure 4.8: Copper restores binding of intact but not of N-terminally truncated purified PrP-AP

N-terminally truncated or intact PrP-AP was purified using phosphonate gel chromatography, as described. Binding to G8 cells was then assayed in the presence or absence of 1 mM Cu^{2+}.


Effect of Cu2+ on PrPAP Binding
OD 620 nm
3.5
3
2.5
2
1.5
1
0.5
0
Truncated
Intact
Untreated
Cu2+

Following the characterization of the binding of PrP-AP to the cell surface, we decided to identify this putative receptor. Given the near ubiquitous expression of the binding activity on the cell lines assayed, we had to develop an expression cloning system other than the ones traditionally used. This chapter describes this novel methodology and the results of the expression cloning screen.

Chapter 5

Expression Cloning of a Prion Protein Receptor in Xenopus Oocytes[1]

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Darwin Molecular#, Bothell, Washington, USA

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Contributions of additional authors

Ms. Margit Gayle was instrumental in instructing me on how to construct the expression library used in this study. She also performed the final sequencing of the isolated clones. Dr. Eustache Paramithiotis aided in the injection of Xenopus oocytes for the screening of the library.

Summary

The normal cellular isoform of the prion protein PrP^C is an evolutionarily well conserved surface glycoprotein of unknown function, linked to the outer leaflet of the membrane via a glycosylphosphatidyl-inositol (GPI) anchor. We have previously demonstrated the widespread expression of a high-affinity cell-surface binding activity for PrP using a prion-alkaline phosphatase fusion protein (PrP-AP). To seek the identity of this potential receptor we constructed a cDNA library derived from the mouse myoblast line G8, which expressed an abundance of PrP-AP binding activity and performed expression cloning using a novel Xenopus oocyte system. Two clones of interest were identified from a pool that conferred binding to injected oocytes: protocadherin 2 (PC2) and cadherin 11. Injection of the isolated PC2 clone but not the cadherin 11 clone conferred significant binding to oocytes. We propose that members of the protocadherin family may interact with PrP at the cell surface.

Introduction

The prion protein is best known as the infectious agent responsible for a class of neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs). The pathology of TSEs is characterized by neuronal loss, spongiform change, gliosis, and accumulation of the abnormal, protease-resistant form of the prion protein, PrP^{Sc}. The prevalent 'protein-only hypothesis' of prion disease proposes that PrP^{Sc} is derived from the normal cellular prion protein, PrP^{C}, and that the pathogenic form can 'replicate' by converting the normal protein to the abnormal (Prusiner, 1991).

Although the role that PrP^{Sc} plays in disease has been extensively characterized, the function of the normal prion protein is unknown. The presence of PrP^{C} on the cell surface of a wide variety of tissues suggests that it may interact with other molecules on the surface of the same or adjacent cells. Work by Harris and colleagues has implied the existence of a 'receptor' for PrP^{C}, responsible for the internalization of the protein through clathrin-coated pits (Shyng et al., 1994; Shyng et al., 1995). Prusiner and colleagues have suggested that a prion binding protein, dubbed 'protein X', modulates the efficiency of disease transmission in transgenic mice models (Telling et al., 1995).

Several candidate proteins have been proposed as PrP binding proteins (Martins et al., 1997; Oesch et al., 1990; Rieger et al., 1997). Although these proteins may interact with PrP in some fashion, none

fully satisfy the requirements of a widely expressed, high affinity cell surface receptor. We previously determined that a prion-alkaline phosphatase fusion protein (PrP-AP) bound specifically and with high affinity to the surface of a wide variety of cell lines (chapters 3 & 4). This binding activity involved a protein component, since protease treatment of target cells virtually ablated binding (chapter 4).

Our objective in this study was to identify the protein(s) with which PrP-AP interacts with high-affinity. The G8 myoblast cell line had been previously shown to express high levels of binding activity and was therefore used to construct a cDNA expression library. Given the near ubiquitous binding of PrP-AP to cell lines, we developed a novel expression cloning screen using Xenopus oocytes, which exhibit little to no binding activity. We identified two clones of interest from a pool that conferred binding to injected oocytes, coding for protocadherin 2 and cadherin 11. Re-injection of the isolated clones demonstrated that only the protocadherin 2 clone conferred statistically significant binding to oocytes. These results suggest that one or more members of this large superfamily may interact with PrP at the cell surface.

Results

Conventional approaches to expression cloning require the use of cell lines that are easily grown, transfect well, and express high levels of the target proteins. In addition, the cells must not express the molecule to be cloned, allowing the transition from negative to positive expression upon transfection of the appropriate DNA. This caveat precluded our use of all the cell lines traditionally used for this technique, given the widespread expression of the receptor for PrP (see Chapters 3 & 4).

We therefore developed a novel expression cloning methodology using Xenopus oocytes, which possess little or no intrinsic PrP-AP surface binding activity. Xenopus oocytes are most often used for ion channel studies since they can be microinjected with target DNA or mRNA and assayed electrophysiologically following protein expression. We adapted this technique for use with cell surface detection using the PrP-AP fusion protein. An outline of the methodology is depicted in Figure 5.1.

We constructed a random-primed cDNA plasmid library from the G8 myoblast cell line, which had been previously shown to express high levels of the PrP receptor (Chapter 4). Plasmid DNA, derived from pools of approximately 2000 clones, was then linearized for in vitro transcription and capping. The resulting mRNA was microinjected into oocytes which were then allowed to recover for a 48 hour time period, previously determined to be optimal for protein expression

using the Elf-1/Mek4-AP ligand receptor pair (see below). Injected oocytes were then assayed colorimetrically for PrP-AP binding using an adaptation of the previously described protocol (described in Methods).

For each experiment, the negative control consisted of oocytes "mock injected" without mRNA. Positive controls were provided by oocytes injected with mRNA prepared from an Elf-1 plasmid clone and detected using a Mek4-AP fusion protein (kind gift of Dr. J.G. Flanagan, Harvard, Cheng and Flanagan, Cell 1994).

Given our previous observations concerning copper as an essential co-factor for the interaction of PrP with its receptor, we performed the binding assays with PrP-AP both in the presence and absence of 1 mM CuSO4. Figure 5.2 shows the results of a typical round of screening, including positive and negative controls.

As seen in Figure 5.2, pool #35 conferred binding to PrP-AP which was enhanced in the presence of copper. Subsequent iterative dilution of pool 35 disclosed that clone 6, encoding a fragment of the mouse homologue of protocadherin-2 (PC2) was responsible for the preponderance of binding conferred by this pool. Clone 7, encoding a fragment of the mouse homologue of cadherin 11 (Cad11), was also found to confer moderate binding of PrP-AP to injected oocytes. However, multiple injection experiments determined that the binding seen with cadherin 11 was not statiscally significant (Figure 5.3).

Discussion

In this study, we have used a novel expression cloning strategy based on Xenopus oocytes to identify a candidate cell-surface receptor for the normal cellular isoform of the prion protein, PrP^C. We identified one clone which conferred statistically significant binding of PrP-AP upon microinjection into oocytes: protocadherin 2. Our data suggest that protocadherin 2 and potentially other members of this large superfamily of molecules may play a role as PrP interacting proteins in addition to their proposed cell-cell adhesion function.

The cadherins were initially recognized as calcium-dependent, homophilic cell-cell adhesion molecules, collaborating with other adhesion molecule families (including the integrins, immunoglobulin family adhesion molecules, and the selectins) in cell-cell recognition and adhesion phenomena in developmental formation and maintenance of tissues (reviewed in (Suzuki, 1996)). The cadherin superfamily comprises a diverse group of proteins defined by possession of extracellular "cadherin motifs" (~110 amino acids), which fold into repeated domains sharing some tertiary structural features of the immunoglobulin fold. By contrast, the cytoplasmic domains of the cadherins markedly diverge, reflecting in part the widely differing functions of these molecules. Sequence considerations suggest that the cadherin superfamily can be considered as at least two sub-superfamilies: the classical cadherins and the protocadherins.

The "classical" cadherins are a family of relatively tissue-specific molecules that mediate compaction in early embryogenesis, cell-cell adhesion in development and maturity, and neurite extension, in addition to many other known activities (reviewed in (Suzuki, 1996)). Classical cadherin effects are thought to be mediated by attachment of the conserved intracellular domain to the catenins, which in turn bind to the cytoskeleton (Figure 5.4) (Hirano et al., 1992).

The protocadherins have proven more mysterious than the classical cadherins. They are a large, incompletely characterized group of proteins with homology to classical cadherins through possession of extracellular cadherin domains. However, the cytoplasmic domains of some family members diverge markedly from those of the classical cadherins, suggesting an interaction with cytoplasmic proteins different from the known catenins (Figure 5.4). The protocadherins may thus have specialized signalling roles distinct from those of the classical cadherins (Sago et al., 1995). Additionally, the EC1 binding motif of the classical cadherins, responsible for the calcium-dependent homophilic adhesion, is absent from the characterized protocadherins. Indeed, transfection of some protocadherins in model cells does not appear to confer calcium dependent adhesive interactions as observed with classical cadherins (Obata et al., 1995; Sano et al., 1993). Thus, the natural ligand(s) for some of the protocadherins are proposed to be heterophilic and currently unknown. It is possible that our screen has identified PrP as such a ligand.

Several proteins have been proposed to interact with PrP, although many are not integral membrane components and thus not likely to constitute true receptors (Oesch, 1994; Yehiely et al., 1997). Recently, two reports have claimed the identification of a receptor for PrP, one being an unknown neuronal protein of 66 kDa and the other the 37 kDa laminin receptor precursor (LRP) (Martins et al., 1997; Rieger et al., 1997). However, neither study provides a measure of the affinity of the proposed interaction or an indication of the functional domains involved. Additionally, the tissue distributions of these proposed receptors does not correlate with that of PrP^C, which would be expected of a cognate receptor. Notably, the 37 kDa LRP is absent from muscle, whereas we have previously found muscle cell lines to have very high receptor expression.

It is difficult at this point to reconcile the non-complementary data, given the large discrepancies and lack of functional analyses. The possibility exists that PrP interacts with several different cell-surface proteins, although our previous work suggests that the high-affinity interaction observed with PrP-AP is limited to a single receptor type. The cadherin/protocadherin family present an attractive option as receptors for PrP given their binding of PrP-AP and their high level of expression in the nervous system as well as a wide variety of peripheral tissues. However, it remains to be determined if one or any of these molecules represent a true functional, high affinity receptor for the normal cellular isoform of the prion protein.

Materials & Methods

Construction of expression library

Total RNA was extracted from G8 cells using Trizol reagent (Gibco BRL, Burlington, Ontario, Canada). An oligo dT cellulose column (Pharmacia, Baie D'Urfé, Québec, Canada) was then used for isolation of mRNA. mRNA was reverse-transcribed to cDNA using the TimeSaver cDNA kit (Pharmacia, Baie D'Urfé, Québec, Canada) according to the manufacturer's instructions. The cDNA was then blunted with T4 DNA polymerase (Gibco BRL, Burlington, Ontario, Canada) and ligated to BamHI adaptors. Unligated adaptors were separated from adaptored cDNA by S-1000 resin (Pharmacia, Baie D'Urfé, Québec, Canada) chromatography. The resulting cDNA was ligated into BamHI digested pCDNA3.1 (Invitrogen, Carlsbad, CA), transformed by electroporation into E.coli, titrated and plated out at approximately 2000 clones/pool. The average insert size was determined to be approximately 1.5 kb.

AP fusion proteins

The construction and expression of the PrP-AP fusion protein was carried out as previously described. The Mek4-AP expression plasmid (kind gift of Dr. J.G. Flanagan, Harvard) was used to generate MEK4-AP conditioned media in identical fashion.

Oocyte preparation

Adult female Xenopus frogs (Boreal, Edmonton, Alberta, Canada) were anaesthetized using 0.2% tricaine methanesulfonate (Sigma, St-

Louis, MO) in water. Small portions of the egg sacks were removed following a minor surgical incision in the lower abdomen. Egg sacks were placed in a collagenase solution (50 mg collagenase type I in 10 mls of 1 x Barth's saline solution (88 mM NaCl, 1 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 $NaHCO_3$, 5 mM Tris-HCl pH 7.4, 1 mM NaPyruvate)) and lightly shaken for 3 to 4 hours for dissociation. Oocytes were then placed in defolliculation solution (100 mM KCl, 2.5 mM $MgCl_2$, 4 mM K_2HPO_4, 6 mM KH_2PO_4, 1 mM EGTA, 1% BSA, pH 6.5) for 10 minutes and triturated gently using a cut and polished pasteur pipet. Oocytes were then returned to 1 x Barth's saline and allowed to recover for 24 hours.

Preparation of mRNA and injection

Plasmid pools were linearized by overnight digest with NotI restriction enzyme, followed by ethanol precipitation. mRNA was transcribed in vitro using the Message Machine kit (Ambion, Austin, TX) according to the manufacturer's instructions. Oocytes were prepared for injection by a10 minute soak in 2x Barth's saline, to allow easier retention of the injection volume. Oocytes were returned to 1x Barth's saline and injected with 50 nl of mRNA solution using a Nanoliter Injector (World Precision Instruments, Sarasota, FL). Injected oocytes were allowed to recover for 48 hours and assayed for binding activity as described below.

Screening of oocytes for PrP receptor expression

Injected oocytes were harvested into a 24 well plate, at 10 to 20 oocytes per well, equal numbers per duplicate. Damaged oocytes

were discarded. Oocytes were rinsed once with 1x phosphate buffered saline (PBS), and then incubated with the appropriate conditioned media for 4 hours at room temperature. Incubations with PrP-AP containing conditioned media were done with and without the addition of 1 mM $CuSO_4$.

Following the incubation, oocytes were collected into 1.5 ml microfuge tubes and washed six times by gentle shaking with cold HBHA buffer (Hank's balanced salt solution with 0.5 mg/ml BSA, 0.1% sodium azide, and 20 mM HEPES, pH 7.0). Excess HBHA buffer was aspirated, oocytes were triturated in Triton X-100 lysis buffer, vortexed and extracted on ice for 15 minutes. Extracts were centrifuged at 12000 x g for 5 minutes and supernatants transferred to a new tube. Extracts were heat inactivated at 65 °C for 15 minutes (to inactivate endogenous phosphatase activity) and re-centrifuged as above. Extracts were then assayed colorimetrically for alkaline phosphatase activity using the BCIP reagent (Kirkegaard & Perry, Gaithersburg, MD). Samples were read at OD 620 nm in an ELISA plate reader.

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Figures

Figure 5.1: Methodology of expression cloning in Xenopus oocytes

Schematic representation of the novel expression cloning strategy developed for expression cloning of PrP-AP binding proteins in Xenopus oocytes

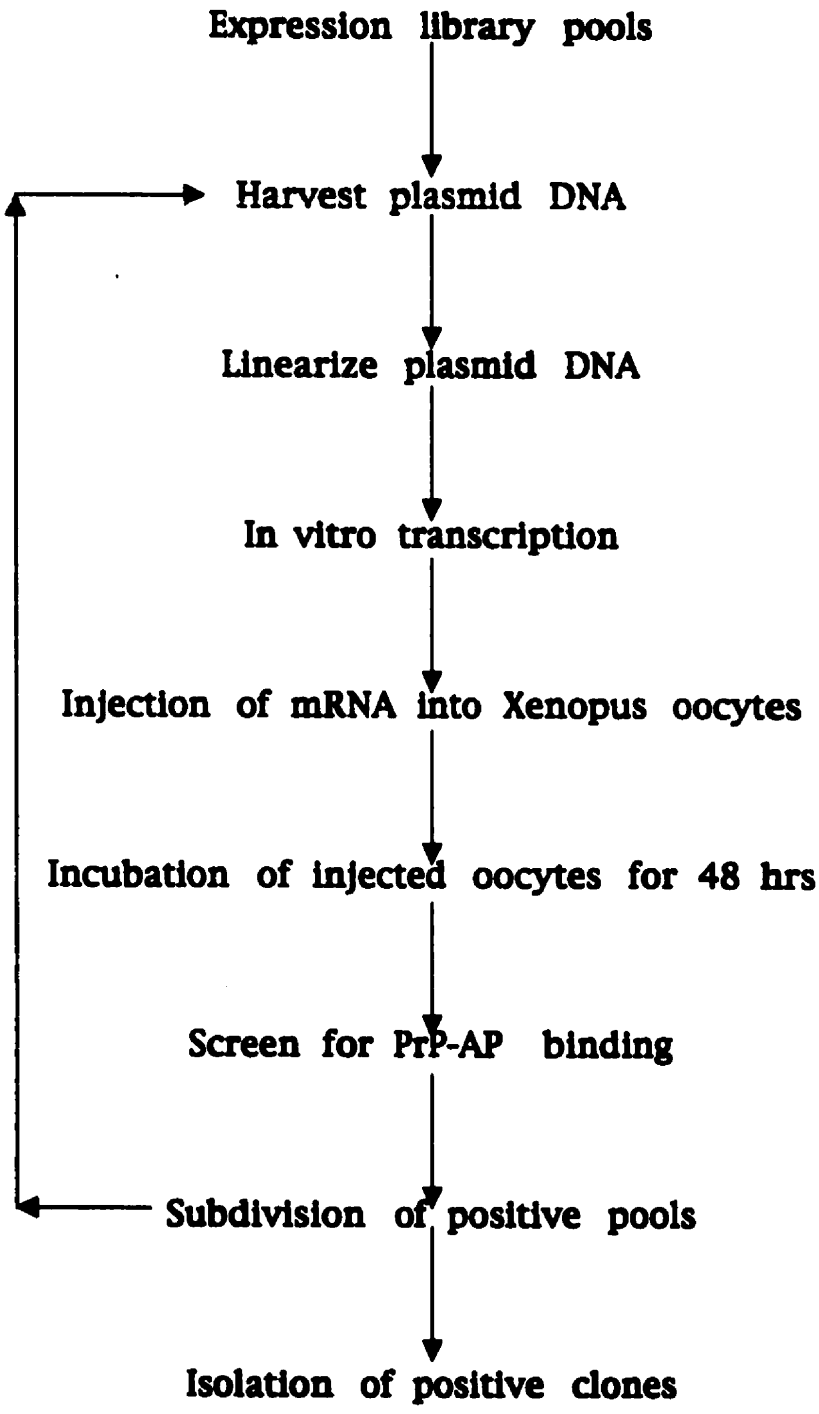

Expression library pools
Harvest plasmid DNA
Linearize plasmid DNA
In vitro transcription
Injection of mRNA into Xenopus oocytes
Incubation of injected oocytes for 48 hrs
Screen for PrP-AP binding
Subdivision of positive pools
Isolation of positive clones

Figure 5.2: Expression screening in Xenopus oocytes

Plasmid pools were linearized, *in vitro* transcribed, and the resulting mRNA microinjected into prepared Xenopus oocytes. Injected oocytes were allowed to recover for 48 hours for optimal protein expression and were the assayed for PrP-AP binding as described. Binding assays were done in the presence and absence of 1 mM $CuSO_4$. Mock injected oocytes are used as negative control.Positive control is provided by oocytes injected with Elf-1 mRNA and assayed with Mek4-AP.

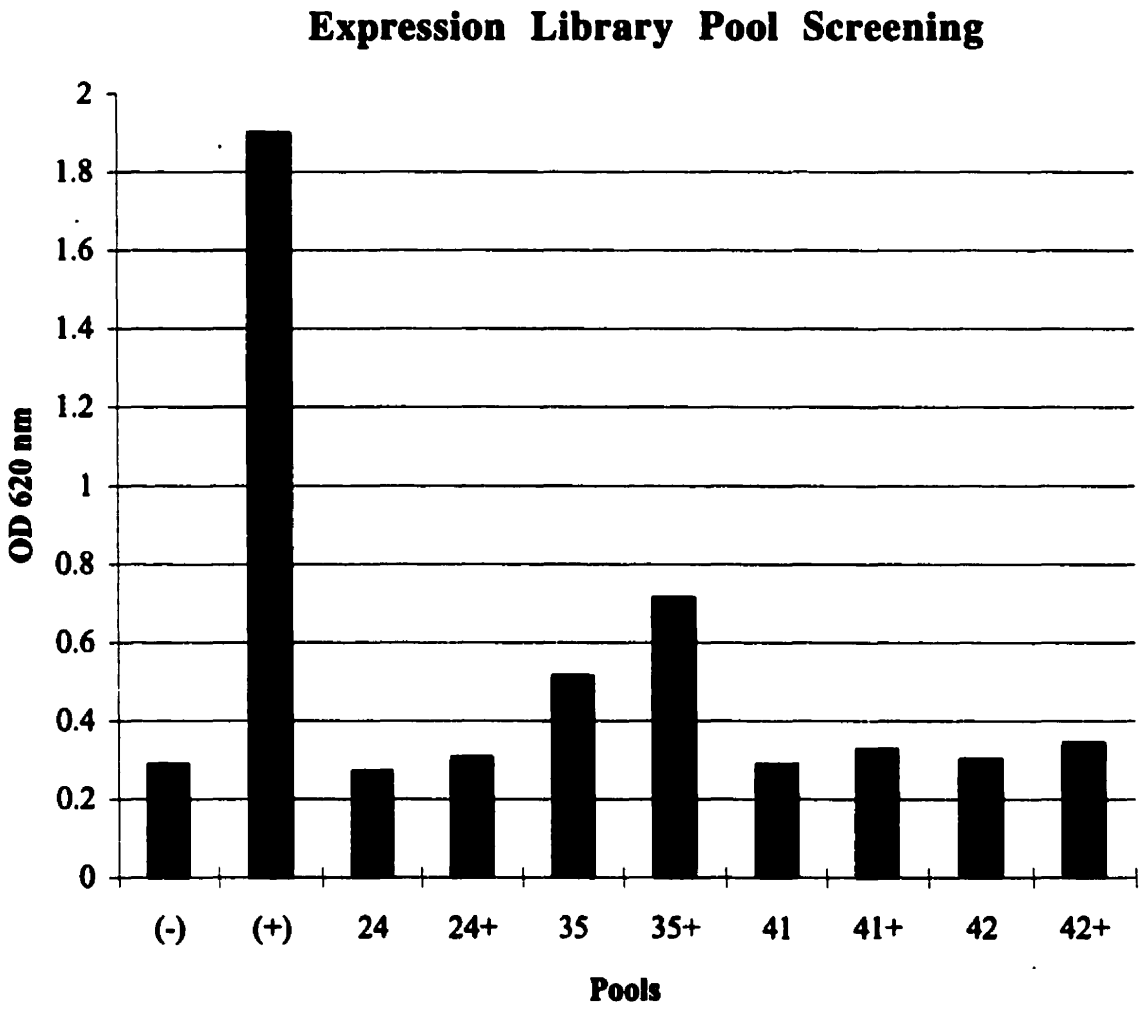

Expression Library Pool Screening
OD 620 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(-)
(+)
24
24+
35
35+
41
41+
42
42+
Pools

Figure 5.3: Protocadherin 2 confers PrP-AP binding to Xenopus oocytes

Isolated clones of protocadherin 2 and cadherin 11 were injected into Xenopus oocytes and assayed for PrP-AP binding as described. Only protocadherin 2 conferred statistically significant binding to injected oocytes.

Protocadherin 2 confers binding of PrP-AP to Xenopus oocytes

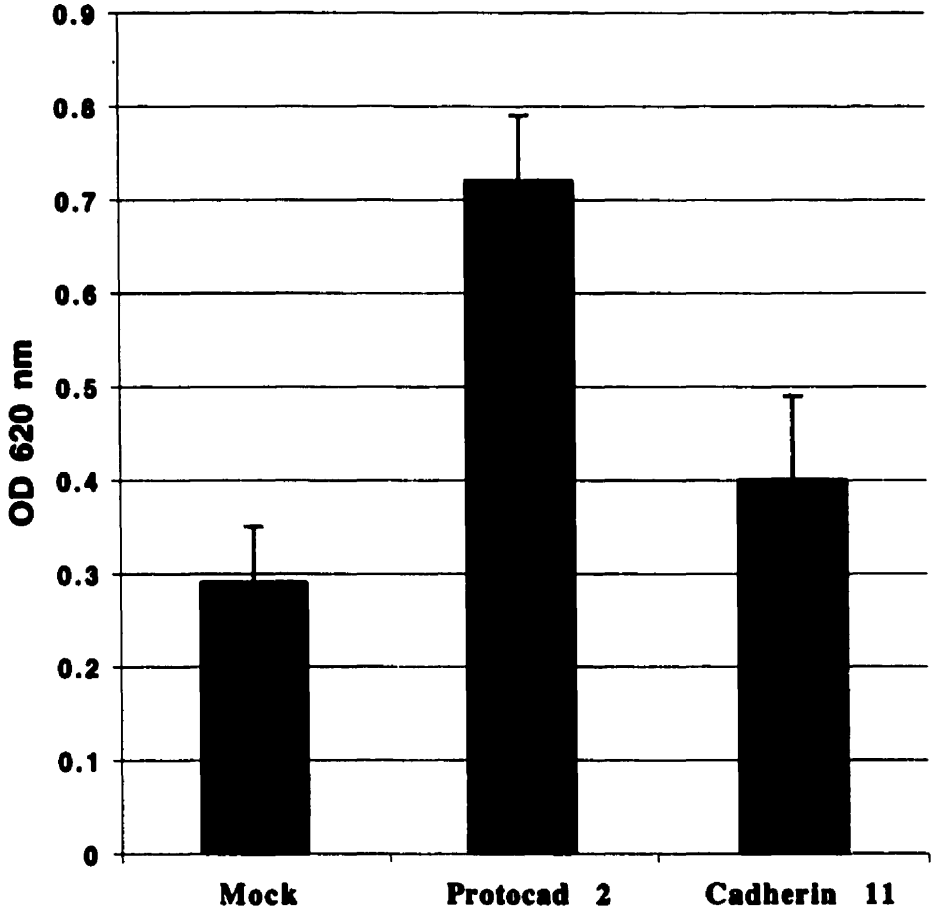
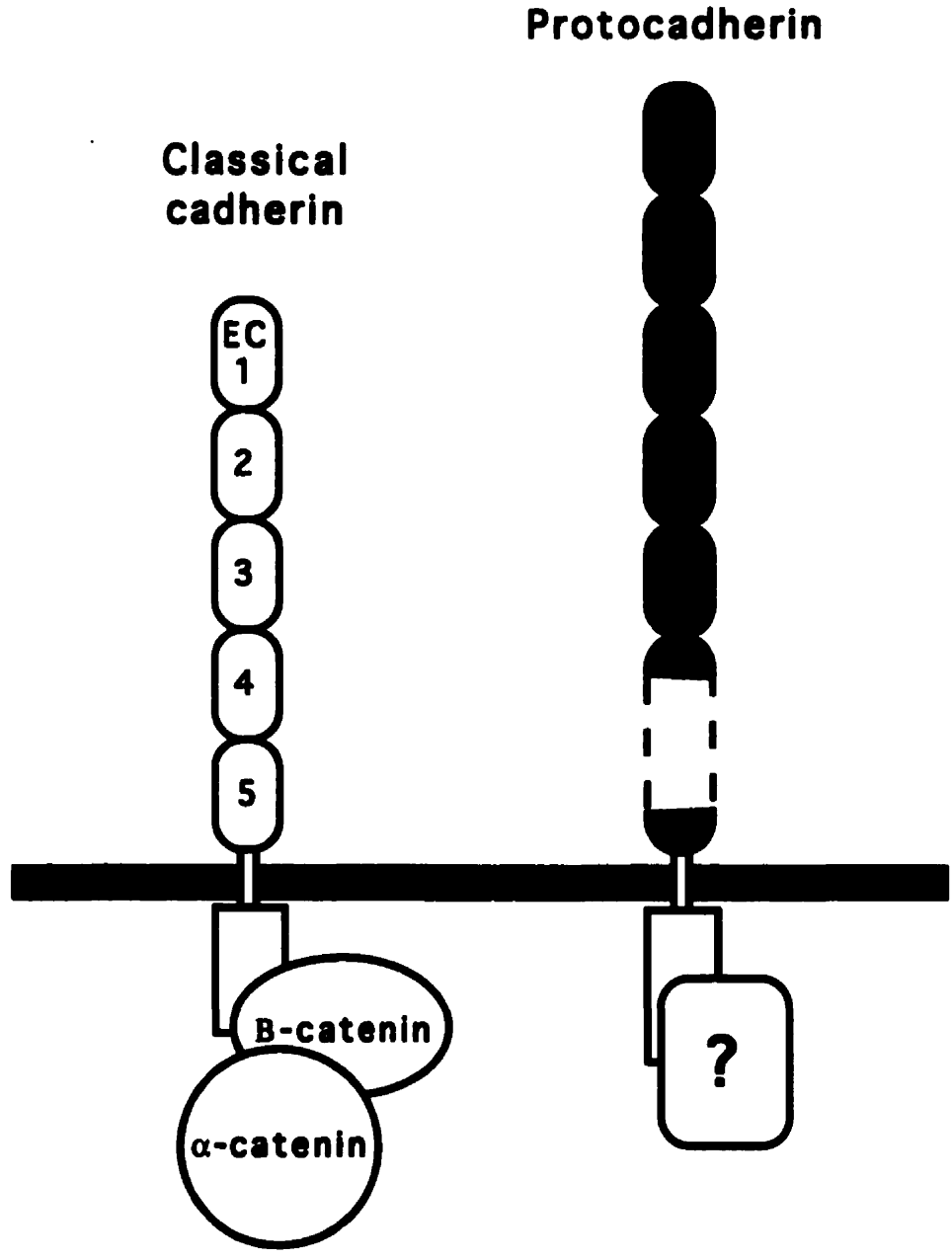


Figure 5.4: Schematic representation of cadherin and protocadherin structure

Classical cadherins are composed of five Extracellular Cadherin repeats (EC1-5). EC1 appears to contain the homophilic cell adhesion domain. Classical cadherins exert their function by association with α and β catenins. The protocadherins do not have an EC1 repeat and associate with uncharacterized cytoplasmic molecules.


Protocadherin
Classical cadherin
EC 1
2
3
4
5
B-catenin
α-catenin
?

Chapter 6
Summary & Conclusions

The data presented in the context of this thesis has focussed on the expression, regulation and function of the normal cellular isoform of the prion protein, PrP^C. We have shown that human bone marrow stem cells express PrP^C, and that PrP^C is then downregulated upon differentiation along the granulocyte lineage, in contrast with the lymphocyte and monocyte lineages. This *in vivo* observation was reproduced using an *in vitro* system of granulocyte lineage differentiation in HL-60 cells. Additionally, the lack of expression of CD15 by lymphocytes strongly suggests the existence of cell-specific glycoforms of PrP^C.

Development of a soluble, alkaline phosphatase tagged form of PrP^C, dubbed PrP-AP, has allowed us to determine that PrP can interact, with specificity and high affinity, with a protein-based determinant on the surface of a multitude of cell lines. We have shown that the N-terminus of PrP^C is intimately involved in this interaction. Binding inhibition studies using synthetic peptides allowed us to map the binding site to the first 11 amino acids of the N-terminus. We have also demonstrated that copper enhances binding of purified and demetalated PrP-AP.

Our desire to determine the identity of this potential receptor for PrP led to the development of a suitable expression cloning strategy. We found that Xenopus oocytes express little to no binding of PrP-AP

and were thus suitable 'hosts' for our expression screen. We had previously shown that the G8 myoblast cell line expressed high levels of binding activity. This cell line was therefore used to construct a cDNA expression library. We identified a positive pool which contained two candidate clones of interest, coding for protocadherin 2 and cadherin-11. Upon re-injection of isolated clones, only protocadherin 2 (PC2) conferred PrP-AP binding to oocytes.

These data address several aspects of prion protein biology. Although tight regulation of PrP^{C} expression at the transcriptional level has been previously observed, little is known of the transcriptional elements involved. Our studies in the granulocyte lineage provide a simple in vitro system for the further analysis of the regulation of the PrP gene. In addition, understanding the reason for the observed down regulation in granulocytes may help elucidate the function of PrP.

Our observation that bone marrow stem cells and certain leukocytes express PrP^{C} is also of consequence for prion disease. Since expression of PrP^{C} is a prerequisite for PrP^{Sc} replication, selected bone marrow cells and leukocytes may support this replication process. This is of concern regarding the risk of transmissibility of prion disease in such medical procedures as bone marrow transplant and blood transfusions. Additionally, the differential expression of the CD15 (SSEA-1) carbohydrate moiety suggests that all PrP^{C} may not be glycosylated in the same fashion. PrP^{Sc} has been found to have

a high proportion of its N-linked glycan chains terminated with CD15 (Endo et al., 1989). The lack of CD15 on lymphocytes precludes its presence on PrP^C expressed by these cells. Differences in glycosylation have recently been implicated in the pathological patterns associated with different prion 'strains' (DeArmond et al., 1997). Thus cell-specific glycoforms may determine cellular susceptibility to infection by the variants of the prion agent.

Although the function of PrP is unknown, its regulated presence on the cell surface strongly suggests a role in cell adhesion, signalling, or uptake of extracellular ligands, all of which imply interactions with other molecules. Our data substantiate the existence of a potential high-affinity receptor for PrP. We have also determined that the N-terminus of PrP is essential for receptor binding. Given that the very N-terminus of the protein is most probably the binding site, the proper presentation of this small polypeptide domain would be crucial in regulating interaction with the receptor. Our findings that addition of copper to a purified fraction of PrP-AP restores binding activity suggests that this metal ion may play a role in conferring a favourable binding conformation to this domain. It has been shown that binding of copper to the octapeptide repeats of PrP induces a shift from the random coil predicted for this region, although Miura et al. report the formation of an α-helical structure in contrast to the β-sheet demonstrated by Stöckel and colleagues (Miura et al., 1996; Stockel et al., 1998).

If copper regulates the interaction of PrP^C with its receptor, it is therefore possible that these two molecules act in concert to regulate, either directly or indirectly, intracellular and extracellular levels of this metal ion. The receptor/PrP^C/copper complex could be internalized, contributing directly to the intracellular pool of copper. Harris and colleagues have demonstrated that endocytosis of chicken PrP^C depends on the presence of an intact N-terminus (Shyng et al., 1995). They have suggested the existence of a receptor that interacts with this domain and targets PrP to clathrin pits for internalization (Harris et al., 1996). Alternatively, the formation of this tripartite complex could serve as a copper sensing mechanism, relaying the information by transduction of an intracellular signal through the receptor, PrP^C, or both. Our laboratory has shown that antibodies to PrP^C can modulate lymphocyte activation, implying an association with a signal transduction pathway (Cashman et al., 1990).

Our proposal that protocadherin 2 and other protocad family members may be a high-affinity receptor for PrP^C is also consistent with the latter option. The cytoplasmic domain of the protocadherins, different from that of the cadherins, has recently been shown to interact with several uncharacterized cytoplasmic proteins (Sago et al., 1995), suggesting the association with novel signal transduction machinery. Additional evidence in the favour of protocadherins as receptors for PrP comes from their widespread expression. The other recently proposed PrP receptor candidates show a tissue distribution that does not entirely reflect PrP distribution (Martins et al., 1997; Rieger et al., 1997). Although a role in cell adhesion has been

proposed for the protocadherins, this activity is very weak relative to the classical cadherins. Along with the signal transduction potential, this suggests that this large, incompletely characterized family of proteins may have a novel function that we propose to be interaction with PrP.

As a final synthesis of the literature and work contained herein, I propose a plausible, although highly speculative, model of the mechanisms at play in prion disease.

This model posits that:

1) PrP^{C} interacts with a cognate receptor, which may be PC2 or a family member, and that this interaction serves to regulate normal copper metabolism.

2) Interaction of PrP^{C} with the disease isoform PrP^{Sc}, disrupts copper metabolism, which contributes directly to the pathology observed in the spongiform encephalopathies.

The proposal that PrP^{C} plays a role in regulation of copper metabolism is reinforced by the observation that brains of PrP null mice have a reduced copper content (Brown et al., 1997a). Cell cultures from these animals exhibit lower levels of copper/zinc dependent superoxide dismutase (Cu/Zn SOD-1) enzyme activity as well as altered electrophysiological responses in the presence of copper (Brown et al., 1997b; Brown et al., 1997a). Additional circumstantial evidence comes from neuronal expression of PrP^{C}, which is highly concentrated in the synaptic cleft. High amounts of copper are released at the synapse during depolarization and are

taken back into the cell by a high affinity copper binding process, perhaps provided by PrP^C and its receptor.

If PrP^C and its receptor regulate copper metabolism, how could this function be involved in prion diseases, and what is the role of PrP^{Sc}? During the disease process, PrP^{Sc} 'recruits' PrP^C to generate an increasingly larger pool of PrP^{Sc}, accumulating up to several ug per gram of brain tissue in rodents at end stage disease. Conversion of PrP^C to PrP^{Sc} may effectively remove functional PrP^C from the cell surface. This loss would disrupt the cell's ability to efficiently regulate levels of free and intracellular copper and thus copper dependent functions, such as Cu/Zn SOD-1 and amine oxidases. Defective copper regulation may be in and of itself toxic to the central nervous system. Treatment of rodents with cuprizone, a copper chelator, has revealed a pathology very similar to that of spongiform encephalopathies with extracellular vacuolation in cerebellar white matter and astrocytic hypertrophy (Kimberlin et al., 1974; Pattison and Jebbett, 1971).

However, PrP^{Sc} may accelerate the process of neuronal damage by acting through microglial cells. PrP^{Sc} has been shown to activate microglia via a non-specific, non-PrP^C dependent mechanism (Giese et al., 1998). These cells then respond by producing reactive oxygen species (ROS). If, concurrently, PrP^{Sc} causes a net loss of PrP^C from neurons, one can envision a feed forward loop where accumulation of PrP^{Sc} increases ROS production by microglia, and PrP^C depleted neurons become increasingly more sensitive to damage by ROS.

Accumulation of free radical damage causes cells to undergo apoptosis, which has been observed to occur in neurons from both scrapie infected animals and fatal familial insomnia affected humans (Dorandeu et al., 1998; Giese et al., 1995). The disease process continues to progress inexorably until the extent of neuronal damage exceeds the capacity to support life functions.

This model also applies to observations in genetically manipulated mice. In PrP null mice, PrP^{Sc} cannot replicate (Bueler et al., 1993) and the original inoculum may be insufficient to activate microglia to any significant extent, with no progression of disease. Mice expressing N-terminally truncated forms of PrP^{C} are susceptible to disease (Fischer et al., 1996). These animals may be functional PrP knockouts with respect to the ability to bind copper. Their neurons would therefore be more sensitive to free radical damage, as has been shown in the PrP null mice (Brown et al., 1997b). PrP^{Sc}, which has been reported to interact with a central portion of PrP^{C} (Kaneko et al., 1997; Telling et al., 1995), would still bind and convert these truncated isoforms to PrP^{Sc}. The ensuing accumulation of the abnormal isoform would result in activation of microglia and production of ROS, which would be highly damaging to the more sensitive neurons of these animals.

Thus PrP^{C} may serve to regulate cellular copper metabolism, and that loss of this function in disease, in addition to potential gain of function by PrP^{Sc}, is a major cause of disease pathology.

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