

Copper, endoproteolytic processing of the prion protein and cell signalling

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1. ABSTRACT

Recently, understanding of many molecular interactions has progressed appreciably and cellular events once thought to be by-products of more important reactions or to be detrimental to cellular function are now known to be part of complex interactions of the cell with its environment. Numerous proteins can elicit differing effects depending upon post-translational modification events such as complex glycosylation and endoproteolytic cleavage or through binding co-factors including metal ions; the prion protein (PrP) is likely one such example. Its absolute requirement for pathogenesis has made the function of PrP an area of intense study but with apparently inconsistent results. This may, in part, stem from the ability of PrP to undergo different modifications to varying extents depending upon precise cellular circumstances. Specific modifications may promote altered association with binding partners resulting in apparent promiscuity of PrP interactions and activation of different signalling pathways, producing the diversity of functions suggested for this protein. This review discusses how modification of PrP by internal cleavage and metal ion co-ordination might influence, or be influenced by, signal transduction cascades.

2. INTRODUCTION

Most mature cellular proteins are not simply the sequence of amino acids encoded by their mRNA translated and folded. Various modifications of the final amino acid sequence occur throughout the biosynthetic process and transit through the cell. Such 'maturation' modifications may include: endoproteolytic cleavage or truncation; addition of non-peptide functional groups (e.g. glycosylation, lipoylation and phosphorylation); co-factor binding; di-sulphide bridge formation; and addition of other polypeptides (e.g. ubiquitination, sumoylation). Extensive studies have described the effects these modifications can have on cell signalling and signal transduction proteins, examples include the requirement of endoproteolytic cleavage for activation of notch and members of the caspase family and numerous enzymes and transcription factors require metal ion binding to facilitate function.

The prion protein (PrP) has been the focus of much investigation since the proposal of the "protein-only" hypothesis for the transmissible spongiform encephalopathies (TSEs) or prion diseases (1). The 'normal' cellular form of the prion protein (PrP^C) has been linked with a signal transduction function since 1990 (2).

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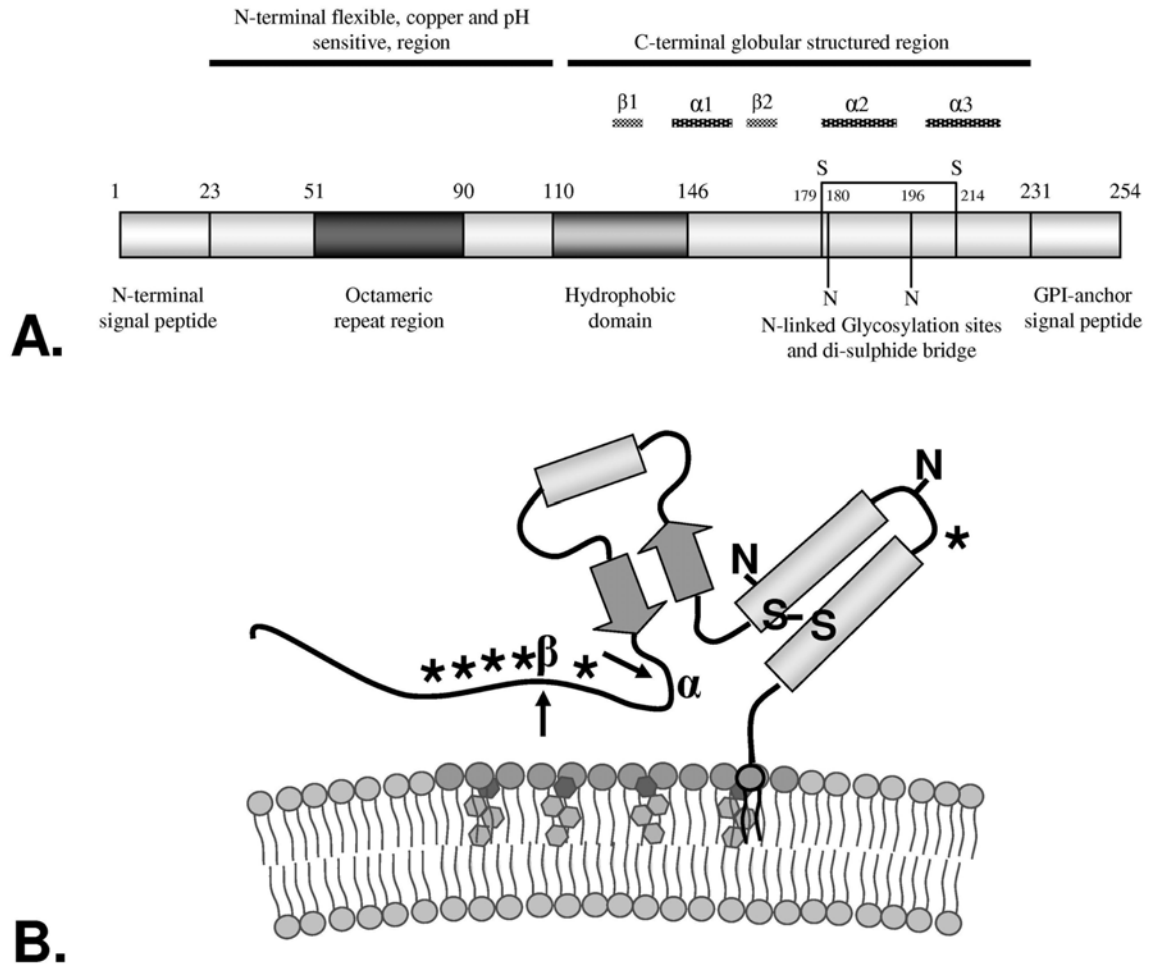


Figure 1. Schematic representations of PrP^C showing locations of structured and functional domains. A. Linear schematic of the PrP precursor protein. B. Diagrammatical representation of PrP^C in cholesterol-rich (hexagon structures) lipid-raft membrane domains. Alpha helices are shown as rectangles and beta sheets as arrows. Copper co-ordination sites are indicated by asterisks and the cleavage sites marked as arrows with alpha (alpha site) and beta (beta site).

This putative feature of PrP^C has been considered in many contexts with various outcomes. In addition to methodological differences, the variation in results is likely to arise, at least in part, due to the complex interplay between the numerous signal transduction pathways of the cell but may also be a function of cell-specific post-translational modifications of PrP^C. PrP^C undergoes several post-translational modifications before it reaches its lipid raft localisation at the cell surface; these are shown in Figure 1. Nascent PrP is co-translationally processed at amino acid 22 to remove its endoplasmic reticulum targeting sequence and at amino acid 231 to remove the glycosylphosphatidylinositol (GPI) anchor signal sequence and allow attachment of the GPI anchor. The di-sulphide bridge forms during folding and complex N-linked glycosylation occurs in the Golgi apparatus before PrP^C reaches the cell surface where its GPI anchor directs it into lipid raft, cholesterol-rich domains. Two further post-translational modifications of PrP^C are recognised as occurring constitutively; di-valent metal ion binding and endoproteolytic cleavage.

To date there have been a number of functions suggested for PrP^C including: copper or metal ion transport and homeostasis (3-5); cellular adhesion (6, 7); protection against oxidative insults (8, 9); lymphocyte activation and proliferation (10, 11); control of circadian rhythms (12) and serotonin homeostasis (13). If validated, the diversity of these functions may partly arise because of an influential role of PrP^C in underlying and overlapping signal transduction pathways. Factors that activate one of the central intermediates of a pathway are also likely to induce secondary activations of other pathways, and the subtleties or nuances in differential activation of signal transduction pathways in different tissues or neuronal sub-populations could result in many outcomes, potentially explaining the diverse functions of PrP^C. Comprehensive background information on central signal transduction pathways has previously been published (14, 15). This review will discuss postulated roles for PrP^C-signalling and related cell functions emphasising the potential involvement of post-translational modifications. In particular, the roles of copper co-ordination and constitutive endoproteolysis of

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PrP^C will be considered as mechanisms by which cells regulate the extent and modulate the nature of PrP^C signalling.

3. PrP^C AND COPPER

3.1. Copper co-ordination by PrP^C

The copper binding sites of PrP^C have been reviewed comprehensively elsewhere (16-18). For the purposes of this review a short overview follows. PrP^C co-ordinates up to four di-valent copper (Cu²⁺) molecules within the N-terminal octameric repeat domain (19-21). It also has two further co-ordination sites between amino acids 90-111, with both histidines 95 and 110 (murine PrP sequence) thought to be essential for co-ordination (termed the fifth site), and putatively another site further into the C-terminal structured domain (22, 23). The octarepeat domain is highly conserved across mammalian and avian species and expansion or contraction of the repeats, which alters copper binding capacity, is associated with hereditary human prion diseases (24). Copper is not the only metal ion that can be co-ordinated by PrP^C; nickel, zinc, and manganese have all been shown to interact with the octarepeat site, albeit with affinity reduced by three or more orders of magnitude as compared with copper, and interaction of these metal ions is even weaker within the fifth binding site (22). Determination of the affinity of Cu²⁺ for PrP^C has been a highly disputed area, with reported affinities that range from femtomolar to micromolar. However, copper binding, as shown by PrP extraction from brain tissue, has been proven *in vivo* (20). The affinity of PrP^C for Cu²⁺ is sufficiently high that Zn²⁺ is unable to compete for the binding sites in full length recombinant PrP or a fragment corresponding to the octarepeat region (25), although, Walter *et al.*, (25) have found that Cu²⁺ co-ordination by full length PrP can be altered in the presence of Zn²⁺. Whilst the amount of Cu²⁺ ions co-ordinated does not change, when less than two equivalents of Cu²⁺ are competed with Zn²⁺, the zinc shifts Cu²⁺ to preferentially occupy the non-octarepeat sites. Over three equivalents of Cu²⁺, zinc has little effect as the higher affinity for copper allows Cu²⁺ to maintain normal binding modes. In short, not only does Cu²⁺ co-ordinate to various locations along the length of PrP, but the preferential nature of those binding locations is altered in a context dependent manner.

Co-ordination of Cu²⁺ within the octarepeat region induces structural changes to the flexible N-terminal region. In the absence of copper binding this region is unstructured in solution; however once Cu²⁺ is co-ordinated the octarepeats assume an alpha-helical structure (26). This is thought to occur due to a stacking effect of the octarepeats by interactions between the nitrogens of the histidine imidazole and the tryptophan of one repeat with the backbone carbonyls of the histidine and glycine of the adjacent repeat (21). Cu²⁺ co-ordination also alters the arrangement of the C-terminal structured region of PrP. Recombinant PrP refolded to reflect the structure of PrP^C, assumes different C-terminal conformations dependant upon whether Cu²⁺ is bound during the folding process or afterward (27, 28).

There is also evidence to suggest that PrP^C is able to co-ordinate monovalent copper (Cu⁺: 29, 30). The sites capable of co-ordinating Cu⁺ are not confirmed, and while initially the octarepeats were suggested to be involved (29) more recently the binding site between amino acids 91-124 was shown to be the only active region in this co-ordination (30). The significance of Cu⁺ co-ordination lies in the ability to more readily undergo redox chemistry. Redox cycle capacity is required for the activity of enzymes such as superoxide dismutase, a proposed function for PrP^C (8).

3.2. Copper and PrP^C function

Copper, whilst essential for certain cellular functions, is highly toxic as a free metal ion and as such cells have evolved intricate systems for ensuring it does not exist in this form long enough to cause cellular damage by redox cycling. PrP^C has been shown to protect against copper toxicity in primary neurones and astrocytes and cell cultures compared with PrP-null counterparts (3, 31). Protection is against the both the production of reactive oxygen species (ROS) and the metal ion itself, probably by preventing erroneous binding to other proteins, and requires the octameric repeat region, although only one repeat needs to be intact for partial protection (31).

Copper saturation of PrP^C extracted from murine brain tissue has been shown to influence its putative antioxidant function. PrP^C only showed superoxide dismutase-like activity when two or more copper molecules per PrP^C molecule were present (9). During prion diseases the metal ion balance of the brain is perturbed with copper levels diminishing and manganese levels increasing (32, 33). This switch in metal ion concentration has been suggested to be involved in prion misfolding (34) but also might considerably change the function of PrP^C. Interestingly, under conditions of decreased intracellular copper, lipid raft copper is seen to rise (35). PrP^C is localised to lipid rafts, cholesterol-rich membrane domains that can function as signalling platforms, and, as a result of having access to a greater pool of copper in its immediate vicinity, might be directly able to respond to the internal deficit by modulating signal transduction.

Transcriptome analysis of human hepatoma cells treated with increased but sub-lethal copper concentrations identified the nuclear factor-kappaB (NF-kappaB) signalling pathway as activated by copper; the authors proposed that this pathway may be involved in cellular survival in response to elevated copper conditions (36). Transgenic PrP^C knock-out mice show few abnormalities, however increased neuronal susceptibility to cellular stress is apparent, and one of the signalling pathways found altered in these mice is the NF-kappaB pathway (37). Knock-out of PrP^C produced increased constitutive and inducible NF-kappaB, increased phosphorylated (active) Extracellular Regulated Kinase (ERK) and increased Bcl2, all of which are involved in cellular protective reactions. Therefore, PrP^C mediated protection against copper and oxidative stress may result from interaction with and modulation of the signal transduction pathways involved in cellular protection and survival.

3.3. PrP, Copper and Signal Transduction

Copper in physiologically relevant concentrations induces rapid internalisation of PrP^C (38-40). Copper-induced internalisation requires the octameric repeat region, the far N-terminal amino acids and the palindromic sequence adjacent to the alpha cleavage site (see section 4.1: 40, 41). Recent evidence has shown that internalisation of PrP^C is essential for activation of signal transduction by stress inducible protein I (STI-1; 42); copper binding may therefore contribute to signal transduction through internalisation of PrP^C. Increased exogenous copper can specifically activate PrP^C mediated p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK, also referred to as stress activated protein kinase, SAPK) signalling in a species specific manner (43). When extracellular copper is depleted, green fluorescent protein (GFP)-tagged PrP^C accumulates at the cell surface; consequently, diminished extracellular copper may abrogate or attenuate signalling through PrP^C by limiting its internalisation (40).

Many of the classical signal transduction intermediates are not free within the cell, requiring a targeting scaffold by which they are mobilised to their site of action. Signalling endosomes have been especially associated with clathrin and dynamin internalisation (44, 45), and these are both pathways with which PrP^C has been shown to interact (46, 47). This link is further supported by the findings of Caetano *et al.*, (42), which showed that signalling by STI1 through ERK1/2 upon internalisation of PrP^C was inhibited by a dominant-negative mutant of dynamin. Further, lipid rafts are involved in endosomal signalling, and lipid raft adaptor proteins are required for anchoring the MEK-ERK signalling intermediates to the cytoplasmic face of late endosomes (48). The lipid raft localisation of PrP^C is also consistent with a potential influential role in this form of signal transduction.

Copper may also modulate PrP^C related signal transduction by altering PrP^C secondary structure. Since the precise fold of the protein will most likely influence the repertoire of potential binding partners, through consequent exposure or burial of epitopes, this could conceivably have ramifications on the specific signal transduction pathway engaged. Although overall C-terminal structure appears altered by binding Cu²⁺, albeit when PrP^C is refolded in the presence of copper, the region most affected is helix 1 (28). Dimerisation of PrP^C through its hydrophobic domain (cited as murine amino acids 113-133) induces activation of signal transduction through ERK and JNK, which is disrupted by the disease associated PrP^C isoform, PrP^{Sc} (49). Helix 1 starts at amino acid 144 of the murine sequence, nearly juxtaposed to this hydrophobic region, and therefore any changes to the structure of helix 1 induced by copper binding may alter the availability of the hydrophobic domain for binding and subsequent signal transduction. Furthermore, Cu²⁺ binding is sufficient to dimerise membrane anchored fragments derived from the PrP^C N-terminus (50). Therefore Cu²⁺-regulation of PrP^C dimerisation through the N-terminus could play an important role in initiating cell signalling at the cell membrane.

4. CLEAVAGE OF PRP

4.1. Characterization of PrP^C cleavage

PrP^C can undergo two unique constitutive endoproteolytic events, which have been termed alpha-cleavage and beta-cleavage. The C-terminal peptide resulting from alpha-cleavage has been termed C1, and the complementary N-terminal peptide N1; the beta-cleavage products are analogously termed C2 and N2. A further cleavage event producing C3 and N3 fragments has recently been identified but little characterisation of this event is available as yet (51). By means of radio-sequencing, the site of the alpha-cleavage event has been located between amino acids 111/112 of the human PrP^C sequence (52). The location of the beta-cleavage event is more variable; the C-terminal product C2 extends from the GPI-anchor to amino acid 90 on average (52, 53) but can include part of the octapeptide repeat region (54, 55).

4.1.1. Enzymatic mediators of alpha-cleavage

The C1 peptide, indicative of alpha-cleavage, has been identified in both normal (healthy control) and CJD brains (52), as well as in various cell-types from diverse species in culture (56), indicating that C1 is a significant product of normal PrP^C processing. This processing appears to have both a constitutive and an inducible component. Induction of alpha-cleavage, correlated by N1 secretion, has been demonstrated by application of phorbol esters (57) and by treatment with a muscarinic receptor (M1 and M3) agonist (58). The phorbol ester and muscarinic receptor stimulated activation of alpha-cleavage appears to be catalyzed completely by a disintegrin and metalloproteinase (ADAM)-17 (57, 58). Another member of the ADAM family, ADAM10, has been shown to have a significant role in the constitutive production of C1/N1 (57). However, whereas knockout of ADAM17 was shown to abolish stimulated N1 secretion, knockout of ADAM10 reduces constitutive cleavage without abolishing it. This is not dissimilar from other substrates of ADAM10/ADAM17. For instance the GPI-anchored cytokine fractalkine (also named CX3CL1) is proteolytically shed constitutively by ADAM10 and in an inducible manner by ADAM17, and yet ADAM10-null cells still maintain partial fractalkine shedding (59). In the case of fractalkine, the partial shedding maintained in ADAM10-null cells may be abolished by ADAM17 inhibition (60), suggesting that while ADAM10 may be the primary constitutive sheddase of fractalkine, ADAM17 is capable of fulfilling that role in the case of ADAM10 knockout or inhibition.

Although ADAM10 and ADAM17 remain the only enzymes positively identified as having a role in PrP^C alpha-cleavage, a recent publication by Taylor *et al.* (61) questions the primacy and extent of their involvement. This study demonstrated that constitutive C1 production was not affected by alterations to expression of ADAM10 or ADAM17. *A priori*, while it was not expected that ADAM17 knockdown should affect basal levels of alpha-cleavage products, given that its role appears to be primarily inducible, the report that neither transfection of ADAM10 nor its knockdown affected C1 levels is

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intriguing. Instead, an *in vitro* test found that recombinant ADAM10 does not cleave PrP^C at the alpha-cleavage site, but rather it acts as a sheddase of PrP, cleaving PrP^C just proximate to the GPI-anchor (61).

Given contention in the reported findings to date, it is difficult to definitively state what role ADAM10 and ADAM17 play in the alpha-cleavage of PrP^C. One reason for the differing *in vitro* results may be the use of HEK cells over-expressing PrP^C (61), compared to other studies which utilized HEK cells expressing only endogenous PrP^C levels (57, 62). Another difference which may account for the varying conclusions may be that whereas both of the studies, which concluded that ADAM10 is involved in alpha-cleavage, measured N1 in the media, Taylor *et al.* (61) measured C1 in cell lysate. It is arguable whether characterising alpha-cleavage by measuring C1 levels would be significantly different from measuring N1 levels, given that the two peptides are inevitably produced in parallel from the same cleavage event. One justification for quantifying alpha-cleavage by reference to C1, as opposed to N1, is that the former is cell-associated whereas the latter has been measured from the media, and so may less accurately reflect the principle product of alpha-cleavage, which occurs in an intracellular compartment. Temperature blocks to modulate PrP^C trafficking show that alpha-cleavage can, and perhaps predominantly does, occur after the endoplasmic reticulum, most likely within the Golgi network (63) and not at the cell surface (62, 64). If in fact the differing conclusions result directly from the choices to measure either of C1 or N1 levels, then it would need to be possible for C1/N1 to be produced simultaneously in parallel yet with independent mechanisms for the cellular release and clearance of N1. For instance, if ADAM10 were capable of inducing N1 secretion from the cell, without being responsible for the actual cleavage which produces N1, then modulating ADAM10 activity would affect extracellular N1 levels without affecting cellular C1/N1 levels. A more straightforward possibility suggested by Taylor *et al.* (61) is that ADAM10 is not the primary alpha-cleavage enzyme for cell-associated PrP^C, at least in certain cell lines, implicating a yet unknown enzyme or enzymes.

One significant contributor to the difficulty faced to date in identifying the precise cast of alpha-cleavage enzymes is that there tends to be a complex redundancy amongst the metalloproteinase family members, as has been elaborated upon in an extensive gain-of-function experiment (65). Although no other specific enzymatic mediators of alpha-cleavage have been confirmed, there has been evidence supporting a role for serine proteases (66), while this suggestion has been dismissed by others (57, 67). A role for the proteasome has also been put forward (68). Given such difficulties, it is probably instructive to compare other ADAM10/ADAM17 substrates for which additional proteolytic enzymes have been found. Recent examples include identification of matrix metalloproteinase (MMP)-2 (69), MMP-9 (70), and calpain (71) as contributing alongside the ADAMs to the shedding of fractalkine, the receptor for advanced glycation end products, and glycoprotein Ib-alpha, respectively. Other

members of the ADAM family may also be involved in PrP^C alpha-cleavage, although aside from ADAM9 which increases the activity of ADAM10 (72), the potential roles of other ADAM proteins have not been investigated. An obvious target is ADAM23 which has recently been proven to significantly co-localise with PrP^C (73).

Another potential difficulty in determining the enzymes primarily responsible for PrP^C alpha-cleavage, is that the alpha-cleavage recognition motif does not appear determined by the specific amino acid sequence (68), suggesting that the major determinant of alpha-cleavage may be contextual topology, perhaps governed by the precise interactions of PrP^C with its microenvironment and binding partners. Amongst those aspects of the microenvironment worth considering in this regard is the presence of PrP^C within lipid rafts, which is particularly relevant due to the ability of rafts to selectively exclude or concentrate proteins; pertinent examples being the normal exclusion of ADAM10 and ADAM17 from lipid rafts (74, 75). Another aspect of a membrane-associated proteins' microenvironment, and one which is yet to be extensively characterized for PrP^C, are tetraspannins, a family of transmembrane proteins that organize novel microdomains (76). There has been only a single reported study into tetraspannins in the vicinity of PrP, showing that intracellular PrP^C and tetraspannin CD63 colocalise (77). Although the functional implications of PrP-CD63 colocalisation have not been examined, other studies have found that particular tetraspannins positively regulate ADAM10-mediated cleavage (78, 79), suggesting that PrP-tetraspannin association could similarly have implications for regulating alpha-cleavage and possibly also for beta-cleavage.

4.1.2. Mechanisms and locations of beta-cleavage

Unlike alpha-cleavage, PrP^C is known to undergo spontaneous beta-cleavage under appropriate conditions, including those present in post-mortem tissues (80). Beta-cleavage can be induced by exogenous application of a reactive oxygen species (ROS, e.g. H₂O₂ or O₂⁻) in the presence of Cu²⁺ (53) or by serum deprivation (81), and similarly recombinant PrP^C undergoes *in vitro* beta-cleavage in the presence of Cu²⁺ (82, 83). The *in vitro* experiments convincingly suggest that beta-cleavage is a PrP^C autocatalytic event, most likely involving a Fenton-type reaction (84). The actual cleavage event appears to occur, primarily, at the cell surface (63, 81).

Notwithstanding that ROS and Cu²⁺ appear to be sufficient conditions for beta-cleavage, it remains to be determined whether other proteins or lipids can act as co-factors under biological conditions. For *in vitro* reactions, the presence of phosphatidylinositol has been shown to increase the efficiency of beta-cleavage (83), suggesting that membrane composition and microenvironments may be important for modulating cellular beta-cleavage and perhaps alter the precise site, thus resulting in the 'ragged' cleavage observed *in vivo*. Insofar as enzymatic contribution to beta-cleavage is concerned, Yadavalli *et al.* (85) reported that various inhibitors of calpain reduced C2 in PrP^{Sc} infected cells, although calpain did not have any

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apparent role on basal C2 production in non-infected cells, the rate of which has been shown to be dependent on basal ROS (81). One explanation for these results is that calpain is not necessary for beta-cleavage, but is nonetheless capable of contributing to the rate of beta-cleavage. It is possible that PrP^{Sc} increases calpain activity (e.g. by contributing to calcium dysregulation; 86-88), which may contribute to beta-cleavage either by direct interaction with PrP, or else by interacting with some other modulator of beta-cleavage.

4.2. Cell signalling and PrP^C cleavage

4.2.1. Signalling pathways leading to PrP^C alpha-cleavage

Appreciating the temporal and mechanistic context of alpha-cleavage, especially when alpha-cleavage is altered downstream of particular signalling cascades, may suggest function. It has already been noted that alpha-cleavage of PrP^C appears to consist of basal and inducible components, and that these may be independently modulated experimentally and, presumably, in response to *in vivo* signalling prompts. It is noteworthy that (i) PrP-function is most often considered primarily in terms of its presence in neuronal cells, and to a lesser extent in terms of its presence in leukocytes, and (ii) that the signature function of those cells, namely neurotransmission and activation by pathogens, concomitantly induce cleavage of ADAM10/17-substrates. The tantalizing suggestion that follows from this is that alpha-cleavage is involved in some capacity in such cellular activities. Although muscarinic receptors-1/3 are the only receptors to date shown to induce PrP^C alpha-cleavage (58), other G-protein coupled receptors (GPCRs) involved in neurotransmission have been shown to induce cleavage of other ADAM10/17-substrates. Examples include the alpha-secretase cleavage of APP induced by stimulation of serotonin receptors and metabotropic glutamate receptors (89). As for leukocyte activation, related effects on PrP^C cleavage remain to be documented, however studies of other ADAM10/17 substrates provide strong precedence for suggesting that alpha-cleavage would be similarly induced in leukocytes (90, 91), or in other cells in response to leukocyte signalling (92, 93). These facts do not imply that PrP^C is a "neurotransmission molecule", but rather highlight the fact that whatever the functional consequences of alpha-cleavage are, they in some way must be simultaneously coherent within the context of neurotransmission and non-neuronal 'activation' or signalling events that up-regulate cleavage.

Although the pathways linking each of these events remain to be definitively proven, following phorbol ester treatment protein kinase C (PKC; 94, 95) appears involved, along with a kinase cascade possibly including ERK (96, 97), contributing to activation of the ROS producing enzyme NADPH oxidase with eventual activation of ADAM17 (98). It is unclear whether GPCR-induced alpha-cleavage similarly pivots on PKC signalling, although there is evidence supporting this conclusion (72). Studies into muscarine receptor induced APP cleavage have pointed towards a more complex signalling pathway involving release of intracellular calcium stores and NF-

kappaB (99, 100), the involvement of which in PrP^C cleavage remains to be investigated. The pathway linking leukocyte activation and protein-cleavage is even less defined, although it too likely terminates in ROS activating ADAM17; whether NADPH oxidase is similarly involved is arguable (90).

The constitutive nature of ADAM10 activity suggests the likelihood of a mode for basal regulation of alpha-cleavage of PrP^C. As a corollary, the modulation of ADAM10 levels, along with modulation of additional unknown constitutive alpha-cleavage enzymes, may play a role in regulating basal C1/N1 levels. ADAM10 levels are known to be regulated by various mechanisms, including at the transcriptional level by a retinoic acid response element (101) and at a translational level by RNA binding proteins (102). In contrast to ADAM10, PrP^C expression is negatively regulated by retinoic acid (101, 103), which is of uncertain significance but may eventually prove to be of importance. The notable differences observed in steady-state cleavage profiles across cell lines (56) suggests a possibly complex regulation of basal alpha-cleavage, via ADAM10 and additional yet to be identified cleavage enzymes, attuned to the specific needs of the particular cell.

4.2.1.1. PrP^C signalling and alpha-cleavage

Included in the various pathways which are capable of stimulating alpha-cleavage enzymes are those initiated by PrP, allowing for PrP^C signalling to modulate its own full-length/cleaved peptide ratios. These pathways have been described in two interesting studies by Pradines *et al.* (104, 105). The first of these induced signalling by PrP^C antibody ligation, finding consequent increased activity of the transcription factor CREB, resulting in reduced MMP-9 and increased tissue inhibitor of metalloproteinase (TIMP)-1 transcription (104). The upregulation of TIMP-1, which is known to inhibit the activity of ADAM10 (106), may be expected to reduce alpha-cleavage. MMP-9, as has already been noted, may be a candidate alpha-cleavage enzyme; MMP-9 too is inhibited by TIMP-1 (107). The other study linking PrP^C signalling and alpha-cleavage showed that antibody ligation of PrP^C induces ADAM17 sheddase activity (105). Although neither of the studies measured C1/N1 levels, the effects induced by PrP^C ligation on the various metalloproteinases suggests that there is potential for such alteration. Further experiments are required to determine the relevance of the studies to *in vivo* alpha-cleavage and clarify whether PrP^C ligation by a binding partner is similarly capable of modulating metalloproteinases with consequent effects on C1/N1 levels.

4.2.2. Linking PrP^C cleavage and cell signalling

Clearly suggesting that PrP^C cleavage may be mechanistically linked to cell signalling, especially kinase cascades, is the observation that basal alpha-cleavage levels inversely correlate with basal MAPK phosphorylation but increase with a concurrent increase in MAPK activation when the lipid-raft environment is perturbed in the presence of copper (43). Although it is not clear whether this indicates that cleavage directly regulates kinase activity, or whether the events occur in parallel but are unrelated. The

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most direct investigation into alpha-cleavage and function to date is the study by Sunyach *et al.* (108) and its follow up study (109), finding that toxicity to the broad kinase inhibitor staurosporine was enhanced in a p53-dependent manner in the presence of full length PrP^C or C1, but not C2, whereas toxicity is reduced in the presence of N1 but not N2. It was shown that PrP^C and C1 are equivalently capable of positively regulating p53 expression, while N1, but not N2, down regulates p53. In a separate study the authors showed that p53 expression controls PrP^C expression by binding to the PrP^C promoter (110), indicating that full-length and C1 positively regulate, while N1 negatively regulates PrP^C expression. It is curious that C1 and N1 should act in opposition to one another, although, perhaps depending upon the lifetimes of these fragments, such a situation allows a cell through alpha-cleavage to fine tune PrP^C expression whilst secretion of N1 would down regulate expression in neighbouring or target cells.

Beta-cleavage of PrP^C as a functional event that contributes to a signalling pathway has been studied even less than alpha-cleavage, quite likely due to lingering impressions of an inherently pathogenic nature (as suggested by its name, which mimics the nomenclature of the APP toxic Aβ fragment). The basis for categorisation of beta-cleavage as primarily pathogenic includes observations that C2 levels are increased in CJD brains (52), that beta-cleavage in the presence of PrP^{Sc} can form a proteinase kinase (PK) resistant form (85), and that by pre-empting the possibility for alpha-cleavage it prevents the cleavage of the neurotoxic and amyloidogenic 106-126 sequence of PrP^C (108, 111). The experimental conditions that have been found to induce beta-cleavage, namely copper, ROS, and serum deprivation, also suggest that rather than being a mediator of CJD pathology, C2/N2 might instead be products of the stressful environment created by prion pathogenesis. Consideration that beta-cleavage is a functional response to cellular stress is supported by the recent evidence showing that treatment with a peptide comprising the N2 sequence, in the presence of copper, ameliorates serum-deprivation induced intracellular ROS (112). As with the effects on p53 observed downstream of alpha-cleavage products, the pathway by which N2 attenuates intracellular ROS production remain to be established, although it apparently depends on interaction with cell surface heparin sulphate proteoglycans (HSPG: 112). A candidate HSPG is glypican-1, which traffics and functionally associates with PrP^C (113, 114). Similarly supporting that beta-cleavage may be a functional response to cellular stress are PrP^C mutations associated with familial prion diseases, which prevent beta-cleavage, and expression of which increases cellular susceptibility to stress (81, 115). Additionally, the fact that beta-cleavage essentially precludes PrP^C signalling to p53, a pathway which is involved in cell senescence and apoptosis (116), implies that it may also ameliorate toxicity by pre-emptively proscribing targeting to this pathway. It should be noted that while beta-cleavage blocking of PrP-p53 signalling may serve as a relevant mechanism for the *in vivo* protective function of beta-cleavage, it is independent of the protective function demonstrated by the

study treating cells with N2, and which employed PrP-null cells (112).

4.2.2.1. Cleavage and PrP^C binding partners

Additional clues as to further effects cleavage may have on PrP^C related signalling may be based on the consequence of proteolysis on the ability of the protein to interact with binding partners. The implications of any changes to binding capacity resulting from cleavage may vary somewhat depending on whether PrP^C is depicted as a receptor or ligand (i.e. whether its role is to initiate or to transduce a response). It is possible that PrP^C acts to a degree like both a receptor and ligand, as indicated by the binding of PrP^C to STI-1 which induces separate internalization pathways for each protein (42). The insights are also influenced by whether the liberated N-terminal peptides, N1 or N2, are still capable of continued association with PrP binding partners or bind novel partners. Influences of the cleavage events on domain availability for binding partner interactions are shown in Figure 2 and summarised in Table 1.

PrP^C binding partners identified to date, and for which there is data on the PrP^C sequence involved in that binding (PrP^C species indicated) include: STI-1, which binds on the C-terminal side of the alpha-cleavage site, at codons (human) 113-129 (117), and so may be similarly capable of binding full length, C1 or C2; neuronal cell adhesion molecule (NCAM), where although there is evidence that binding to PrP^C is partly affected by the presence of sequence 23-88, it is the C-terminal (of mouse-hamster chimera) 141-176 located well within both C1/C2 which appears most necessary (6); and the N-terminus of PrP^C is sufficient for binding to lipoprotein receptor-related protein-1 (LRP-1), specifically within sequence (mouse) 23-107 which is included within the N1 sequence (118). Finer mapping is necessary to state whether N2, or even possibly C2, contains the LRP-1 binding motif. Another putative associate of PrP^C is tubulin, which interacts with the N-terminus, likely at (human) 23-32 (119), and so would clearly be capable of interacting with either of the N-terminal fragments N1 or N2. PrP^C association with the laminin receptor involves a primary binding site at (human) 144-179 and a secondary site at 53-93 (120), suggesting that each of full length, C-terminal and N-terminal fragments may be capable of binding this receptor.

As alluded to, it is not clear whether, even if harbouring the appropriate sequence motif, the N-terminal fragments retain functional binding equivalent to that which occurs with full-length PrP^C. Additionally, the N-terminal fragments may be liberated to engage binding partners not available to full-length PrP^C. Another consideration is the potentially shorter half-life of these peptides as compared to their full length correlates, as cells often have specialized mechanisms for degrading signalling molecules, avoiding detrimental over-activity (121). If N1/N2 are in fact capable of ligating LRP-1 and/or the laminin receptor, and if this activation is in some sense more efficient at activating those receptors than full length PrP, then it would be the case that PrP-cleavage would, by virtue of the N-terminal fragments, induce ERK/Akt

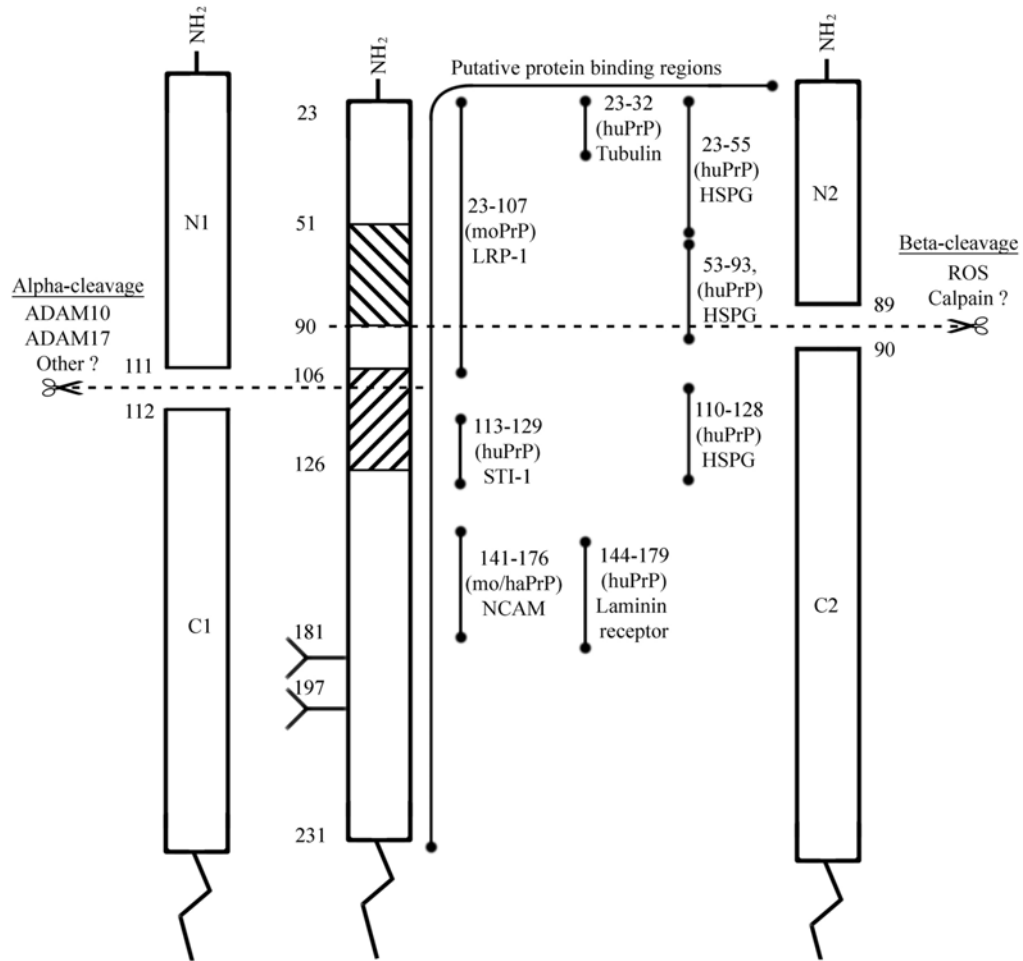


Figure 2. Diagram of human PrP, showing major topographical features, putative protein binding regions, and alpha-/beta-cleavage sites and products. The centre molecule depicts full-length PrP with the fragments formed by alpha cleavage shown on the left and the fragments created by beta cleavage on the right of the full length molecule. Site of interest indicated are the potential glycosylation sites (181/197), the hydrophobic and neurotoxic domain (106-126), and the octapeptide repeat (51-90). Protein binding partners indicate the region of PrP containing the binding site, species of animal sequence is indicated in bracket (including human [huPrP], mouse [moPrP], mouse-hamster chimera [mo/haPrP]).

signalling (122) and/or phosphatase signalling (123) respectively. If N1/N2 are incapable of activating LRP-1 or the laminin receptor independently of the C-terminal domains, then PrP-cleavage may be considered to attenuate LRP-1/laminin receptor signalling. It should be noted that while the ability of PrP^C to bind to LRP-1 and the laminin receptor has been documented, the consequence of binding on signalling cascades are yet to be established. It is of interest that the same ADAM enzymes that catalyse PrP^C alpha-cleavage have also been shown to cleave LRP-1 (124); admittedly this may be no more than coincidence.

Based on putative binding sequences, it is possible that signalling involving STI-1 or NCAM is not affected by either alpha- or beta-cleavage events. Conversely, the loss of the N-terminal domain, following cleavage, may modulate the protein-protein or other interactions, and only permit blockade or non-functional binding of C1/C2 to STI-1/NCAM. This possibility is

suggested by the binding of STI-1 to PrP^C, which induces ERK signalling, and is dependent on the PrP N-terminal sequence despite this being outside the direct binding region (42).

4.3. Cleavage and prion transmissibility

A final consideration for the outcome or consequence of cleavage is how it relates to the propagation of PrP^{Sc}. Whilst the beta-cleavage event has been mainly associated with disease, the aforementioned emerging data suggest an increase in beta-cleavage may at the least also have a parallel protective effect against intracellular stress induced by chronic PrP^{Sc} propagation. The alpha-cleavage event is evident in most standard cell lines employed for study of PrP^C function and the propagation of PrP^{Sc}. In many cases this cleavage accounts for over fifty per cent of the total cellular PrP^C (56). Heightened alpha-cleavage as a proportion of total PrP^C, has been shown to correlate with protection against chronic

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Table 1. Predicted binding partners to PrP alpha- and beta-cleavage products

PrP ^c binding partner	Predicted C1 binding	Predicted N1 binding	Predicted C2 binding	Predicted N2 binding
Laminin receptor	Yes	Yes	Yes	Yes
LRP-1	No	No	?	?
NCAM	Yes	No	Yes	No
STI-1	Yes	No	Yes	No
Tubulin	No	Yes	No	Yes

Characterisation of PrP sequences involved in associations with binding partners is the basis for predicting analogous binding to PrP cleavage fragments. Question marks (?) indicates that there is insufficient information present for predicting binding. See main text for acronym definitions

prion infection in several cell lines, including two cell lines derived from the same GT1-7 precursor, but showing features of divergence over time, with differing C1 cleavage profiles: those cells producing the highest C1 demonstrate resistance to chronic infection (56). Whilst this may be due to the truncated C1 species acting as a suboptimal substrate for conversion to PrP^{Sc}, the alpha-cleavage event has also been linked with signal transduction through ERK and, in addition, through p38 and JNK upon lipid-raft disruption in the presence of copper (43). Inhibition of the ERK upstream activator, MEK, has been shown to reduce PrP^{Sc} load in infected GT1-7 cells (125), while in contrast, inhibitors of the p38 pathway promote PrP^{Sc} formation (126). This suggests that the inverse correlation described between alpha-cleavage and reduced susceptibility to chronic PrP^{Sc} propagation may not just be a result of less full-length PrP^C available as a substrate, but also be due to modulating signal transduction pathways in such a way that the ensuing cellular environment does not favour conversion.

5. PrP^C, COPPER, CLEAVAGE AND SIGNALLING

5.1. Copper and cleavage functioning as interacting contextual switches

The variable presence of copper in the cellular environment and the propensity of copper to bind to various sites along the length of PrP^C suggests a mechanism for directly sensing and responding to changes in the neighbouring milieu. Similarly, environmental cues which alter the basal cleavage of PrP^C may pre-emptively modulate its capacity to initiate signalling and interact with its signalling partners. As has been suggested earlier, some of the apparently paradoxical complexity of signalling pathways in relation to PrP^C function may be the result of failure to adequately delineate the context of those signalling pathways. With this in mind, copper and endoproteolytic cleavage may function as contextual switches, which alter the propensity or capacity of PrP^C to induce any number of signalling pathways. Moreover, each of these contextual switches can be appreciated to alter the influence of the other, as well as further modifying overall resultant function.

The connection between copper and endoproteolytic cleavage is clearest for beta-cleavage, for which there is evidence that copper binding, most likely to the octapeptide repeat domain, directly catalyzes a Fenton-type reaction, which auto-catalytically cleaves PrP^C (84). In the case of alpha-cleavage, Cu²⁺ has been observed to correlate with proteolysis (43), although not at lower

concentrations (81) and during prion disease a high copper diet appears to preclude alpha cleavage favouring beta cleavage instead (33). The mechanism underlying the link between copper and alpha-cleavage remains speculative, although it parallels a similar study which found copper treatment increased alpha-secretase cleavage of APP (127). It is unlikely that copper directly acts on the alpha-cleavage enzymes as most metalloproteinases including ADAM10/17, depend on Zn²⁺ for their catalytic activity not copper (106).

Broadly speaking, there are a number of possibilities underlying the effect of copper binding on alpha-cleavage such as: that it directly results from copper binding to PrP; that it results from copper inducing cell signalling; or that it results from copper modulating the microenvironment of PrP^C. An effect depending on PrP^C binding copper could be the result of altered PrP^C conformation (19). If this is the case it would explain why methionines around the alpha-cleavage site simultaneously modulate the effect copper has on PrP^C conformation and the ability of copper to induce alpha-cleavage (43, 128). Specifically, it may be the case that the presence of a methionine residue regulates the changes in conformation sustained by PrP^C upon copper binding, and that this difference to consequent conformation results in a different propensity of PrP^C to undergo alpha-cleavage.

Insofar as copper modulating alpha-cleavage via a signalling pathway is concerned, it is known that various metals can activate cell signalling pathways (129). Copper has been shown to activate kinase cascades involving phosphatidylinositol-3-kinase (PI3K) and Akt (130), which could potentially act upstream of metalloproteinases (131, 132). If the pathway linking copper and PI3K is responsible for inducing alpha-cleavage, then it is notable that PrP^C is significantly responsible for the extent of PI3K activity in a copper dependent manner (133). Placing these facts into a hypothetical model suggests that copper binding to PrP^C results in PI3K/Akt signalling, which in turn can lead to alpha-cleavage of PrP^C. The possibility, that copper affects the microenvironment of PrP^C, could involve disruption of the PrP-residency within lipid rafts. Recently it was shown that copper treatment results in displacement of flotillin, a protein often employed as a marker for lipid rafts (35) and which is known to associate with PrP^C and be involved in PrP^C signal transduction (134), providing a precedent for copper-modulation of lipid raft composition with potential for secondary effects on PrP^C and on cleavage through the altered microdomain environment of the raft. The potential interactions of PrP and signalling

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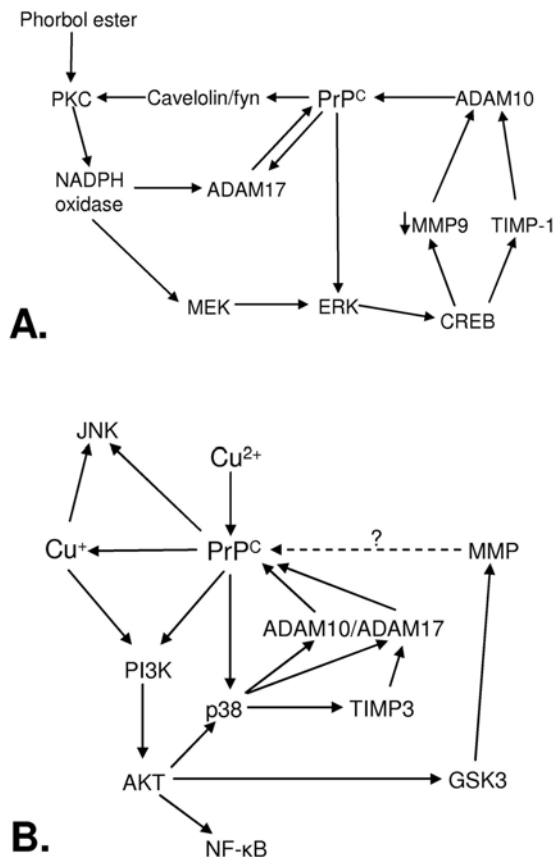


Figure 3. Signalling pathways linked with PrP and potential cleavage enzymes. A. Signalling pathways influenced by PrP that might alter candidate alpha cleavage enzymes. B. The influence of copper on potential PrP signalling pathways and links with signalling pathways activating candidate cleavage enzymes.

pathways with cleavage enzymes and the possible influence of copper are shown in Figure 3.

5.2. PrP, acting as a copper ionophore, influences cleavage and function

The ability of PrP^C to redox cycle copper between the Cu^{2+} and Cu^+ valences might influence signal transduction through the release of Cu^+ inside the cell. Delivery of Cu^+ to the inside of cells using Bis(thiosemicarbazonato) complexes has been shown to up-regulate expression of matrix metalloproteinases by signal transduction through PI3K, Akt, JNK and GSK3 resulting in decreased accumulation of Abeta (135). Addition of Cu^{2+} to the extracellular environment has also been shown to activate this pathway, although it is thought that Cu^{2+} must be internalised and reduced to initiate such signalling (136). Delivery of copper to the inside of the cell via PrP^C might activate similar pathways to those described above, thus regulating cleavage of cell surface proteins, possibly including PrP^C itself. Since the fifth copper binding site is most associated with redox cycling, cleavage and especially the site of PrP^C cleavage, could influence the potential for such activation significantly. By this logic only the N1 and

C2 fragments theoretically could release Cu^+ and initiate signal transduction down these pathways. Akt is known to protect against cell death through the apoptosis inhibitor protein XIAP or by inhibiting the dissociation of Bad and the 14-3-3 protein, and so activation of this pathway by PrP^C delivering copper inside the cell offers a mechanism for a PrP^C related protective function. Recently, the N1 fragment has been shown to be protective against stress insults both *in vitro* and *in vivo* (109) and one mode of action of this peptide might be the intracellular release of Cu^+ .

Alpha-cleavage of PrP^C creates fragments with different copper binding capacity. It is possible that the availability, and consequent copper-loading, of these peptides influences the resultant actions of the cleavage fragments. Another factor worth contemplation is how cleavage alters Cu^{2+} co-ordination by PrP^C, which in turn may influence the C-terminal structured regions (27). The alpha-cleavage event cuts the protein around the end of the fifth binding site, and hence leaves the C-terminus devoid of the most characterised Cu^{2+} binding sites, whereas the beta-cleavage event occurs around the end of the octarepeat region and so leaves the fifth binding site intact. This may have distinct functional implications for both the N and C-terminal cleavage fragments. Removing the main copper-binding domains will likely attenuate the ability of the N-terminus to influence the structure of the C-terminal domain, which may significantly alter the activity of the C-terminus. Further, the influence of zinc on the co-ordination of Cu^{2+} at low occupancy will be altered by cleavage events. The N2 fragment does not contain the fifth binding site and so copper cannot be shifted into this co-ordination mode in the presence of zinc, while the N1 fragment may still retain the ability to shuffle copper between the octarepeat and the non-octarepeat sites. Whilst the exact function of these N-terminal fragments is yet to be determined, copper and possibly zinc may function as important biosensors for modulating downstream effects.

5.3. Copper as a biosensor for cleavage fragment activation

As alluded to, the role of copper in signalling may extend beyond indirectly modulating the secondary structure of the PrP^C C-terminus or activation of pathways by its release into the cell. Copper binding may be integral for the neuroprotective function of the N2 fragment. The ability of exogenous N2 to reduce intracellular ROS induced by serum deprivation requires the binding of two or more copper ions (112). This is consistent with previous data showing that at least two copper ions must co-ordinate to PrP^C for a superoxide dismutase-like function (9). Copper co-ordination also significantly altered the association of N2 with the cell membrane and increased its stability, possibly because of a more controlled association with specific cellular receptors or binding partners. *In vivo*, the absence of the octapeptide repeat region, as engineered into C4 transgenic mice, renders animals more susceptible to larger ischemic infarct similar to the level observed in PrP^C null mice (55). Notably, these mice are unable to undergo beta-cleavage but both the C4 octarepeat-negative and wild type mice show increased amounts of the C1

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fragment. The results are in keeping with N1 having neuroprotective properties which depend on the octarepeat region, or else that the neuroprotective effect observed is dependent on beta-cleavage, and hence likely on the activities of N2. In either case, it is likely that the endoproteolytic and copper binding properties of PrP^C are mutually required for switching on at least some PrP^C-associated neuroprotective functions.

The protective effect of the N2 fragment against heightened intracellular ROS also required cell-surface HSPGs (112). PrP^C has been shown to bind HSPGs via the N-terminal polybasic region (amino acids 23-28), the octameric repeat region and amino acids 110-128 (137). The involvement of the octapeptide repeat region in such binding indicates that copper could significantly influence HSPG binding and it has been suggested that copper could form a bridge between PrP^C and glycosaminoglycans (GAGs: 138). Of the HSPG binding sites identified, the N-terminal site and the octapeptide repeat site have additionally been found to mediate PrP^C binding to the 37 kDa/67 kDa laminin receptor in the presence of heparan sulphate (120, 139). As such, after endoproteolysis, only the N-terminal cleavage fragments N1 and N2 are likely to be able to elicit an effect from 37 kDa/67 kDa laminin receptor binding, with the two main HSPG binding sites lacking in the C-terminal fragments; however, the identification of a C-terminal binding site may mean that some HSPG binding can still occur independent of the N-terminus and octarepeats. Further, the region between the cleavage sites (amino acids 90-111) is potentially important. This region contains a polybasic domain of similar composition to that at the far N-terminus, and so could assume some of the roles of the N-terminal polybasic region following beta-cleavage, including GAG binding capacity.

An additional mode of protection by the N1 fragment against stress could arise from internalisation of HSPGs following binding of the copper-saturated fragment. PrP^C is known to traffic through the cell with glypican 1 (113) and this association has been shown important for conversion into PrP^{Sc} (114). Removal of cell surface heparan sulphate has been shown to increase ADAM17 activity, thereby increasing cleavage of the ErbB4 receptor (140), which has a role in promoting cell survival. Such activation of ADAM17 may similarly increase processing of PrP^C thus producing a self-regulating positive feedback cycle whereby copper-loaded N-terminal fragments internalise HSPGs allowing increased metalloproteinase activity. Variations in heparin sulphate biosynthesis are capable of diametrically altering copper-induced signalling pathways (141), underlying the potential relevance of HSPGs to N terminal fragment copper signalling.

5.4. PrP^C regulation by cleavage products

The control of PrP^C expression at the genetic level is complex, involving not only the promoter region but also control elements in exon 1 and intron 1 and, further, is modified by existing PrP^C expression levels. The regulatory contribution of these control elements varies in cells derived from different tissues (142). The promoter

region itself contains metal response elements with contradictory reports on whether copper up- or down-regulates PrP^C expression (143, 144). However, there is consensus that copper does alter the transcriptional levels of PrP, probably to modulate the function of cellular PrP^C. How copper or other metal ions effect this alteration is still not fully established; activation could be the result of non-specific cellular changes aimed at all proteins with appropriate promoter response elements or could be directly transduced through PrP^C itself.

PrP^C directly influencing its own transcription is suggested by evidence from Gu *et al.* (145) that PrP^C contains two nuclear localisation signals, one located at the far N-terminus (amino acids 23-28, also the GAG binding domain) and the second in the charged domain between amino acids 101 and 112 of the human sequence. These sequences were capable of actively transporting the N-terminus of PrP^C to the nucleus but not full-length PrP^C or fragments extending as far as the first N-linked glycosylation site. Truncated PrP fragments derived from two disease associated mutations with stop codons at amino acids 145 and 160 are also transported into the nucleus in this manner. Therefore the cleavage events producing the N1 and N2 fragments may allow the nuclear localisation sequences to direct these fragments into the nucleus where they could directly alter RNA transcription by acting as transcription factors. Analogous transcriptional activity has been reported for the copper chaperone Atox1; this protein undergoes copper-dependent dimerisation and is directed into the nucleus by a polybasic domain (146).

6. CLOSING CORRELATIVE REMARKS

PrP^C remains an enigmatic protein with apparently many facets. Unravelling its apparently promiscuous activity in various signal transduction pathways, possibly determined by the interplay between its post-translation modifications and the specific underlying cell type, will likely provide insights into this multifunctionality. Interesting parallels can be drawn between PrP^C and insulin-like growth factor 1. This peptide growth hormone is post-translationally modified with complex glycosylation and cleavage liberates the active hormone in a tissue-specific manner. The signal transduction pathways activated are also tissue specific. Insulin-like growth factor 1 displays a predominant circulating form in the blood but shorter N or C terminal fragments are biologically active and have potent neuroprotective function (147). PrP^C shows markedly differing glycosylation and cleavage profiles in cell lines derived from different origins (55), which may hold similar implications. Another interesting signal transduction protein is the Nogo-66 Receptor (NgR). NgR is membrane GPI-anchored and constitutively cleaved during its trafficking to the cell surface (148), which is suggested to occur for PrP^C (63). The contextual variations in activities seen amongst the aforementioned signalling proteins in different tissues may provide insights into why PrP^C functions currently appear so diverse. The parallels between the post-translation modifications of these signalling molecules and those by which PrP^C is processed

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are notable, but establishing their true significance requires further investigation. Cleavage of PrP^C at any of its recognised internal cleavage sites produces fragments of overlapping but distinct molecular qualities, with copper co-ordination being one of the most altered features. The exclusion of cleavage enzymes from lipid raft domains, to which PrP^C is targeted, or inactivation of these enzymes when co-sequestered in lipid rafts, might indicate distinct roles for full-length PrP^C and its cleavage fragments. Copper (and possibly other metal ions) appears likely to be important for PrP^C function, with roles in regulating cellular trafficking from lipid rafts and endoproteolytic cleavage perhaps part of a molecular switch for activation, or possibly deactivation, of signalling with potentially numerous different down-stream responses.

7. ACKNOWLEDGMENTS

SJC is supported by an NH&MRC Program Grant (Identification# 400202) and an NH&MRC Practitioner Fellowship (Identification# 400183); SYM is supported by a Faculty of Medicine Dentistry and Health Sciences, Ronald John Gleghorn Scholarship, the University of Melbourne.

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Abbreviations: PrP: Prion Protein; ADAM: A Disintegrin And Metalloprotease; GPI: glycosphosphatidylinositol; ERK: extracellular regulated protein kinase; MAPK: mitogen activated protein kinase; STI1: stress inducible protein 1; MMP: matrix metalloprotease; PKC: protein kinase C; ROS: reactive oxygen species; APP: amyloid precursor protein; CJD: Creutzfeldt-Jakob disease; HSPG: heparan sulphate proteoglycan; GAG: glycosaminoglycan; JNK: c-Jun N-terminal kinase.

Key Words Prion, Endoproteolytic cleavage, Copper, Signal transduction, Review

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