α-Cleavage of cellular prion protein

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Keywords: prion protein, protein processing, ADAM8, ADAM10, ADAM17, ADAM9, plasminogen, alpha-cleavage, C1 fragment, N1 fragment

The cellular prion protein (PrPC) is subjected to various processing under physiological and pathological conditions, of which the α-cleavage within the central hydrophobic domain not only disrupts a region critical for both PrP toxicity and PrPC to PrPSc conversion but also produces the N1 fragment that is neuroprotective and the C1 fragment that enhances the pro-apoptotic effect of stauroporine in one report and inhibits prion in another. The proteases responsible for the α-cleavage of PrPC are controversial. The effect of ADAM10, ADAM17, and ADAM9 on N1 secretion clearly indicates their involvement in the α-cleavage of PrPC, but there has been no report of direct PrPC α-cleavage activity with any of the three ADAMs in a purified protein form. We demonstrated that, in muscle cells, ADAM8 is the primary protease for the α-cleavage of PrPC, but another unidentified protease(s) also play a minor role. We also found that PrPC regulates ADAM8 expression, suggesting that a close examination on the relationships between PrPC and its processing enzymes may reveal novel roles and underlying mechanisms for PrPC in non-prion diseases such as asthma and cancer.

The Prion Protein (PrP)

Prion protein (PrP), also known as CD230 (cluster of differentiation 230), is encoded by a highly conserved single-copy gene, which is located on the short arm of chromosome 20 in humans.¹⁴ The normal cellular isoform of PrP (denoted PrPC) is highly expressed in the nervous tissues, such as brain and spinal cord, but most other cells and tissues, such as lymphocytes, muscles, heart, digestive track and skin also express PrPαC at lower levels.³ Mature PrPC is a small glycoprotein attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, containing three α-helical regions and two short β-strands, two N-linked glycans (CHO), and a disulfide bridge between the second and third helices (Fig. 1). The flexible N-terminal half contains the octapeptide repeats that bind divalent cations such as Cu²⁺ (Fig. 1).

The PrP protein plays a central role in prion diseases (also named transmissible spongiform encephalopathies), a group of mostly transmissible neurodegenerative disorders represented by Creutzfeldt-Jakob disease (CJD) in humans and by scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD) in animals.³ PrPC is rich in α-helices (43%) and poor in β-sheets (3%), detergent soluble and protease-sensitive. In prion-affected humans and animals, PrPC is converted to the pathogenic scrapie isoform (denoted PrPSc), which is rich in β-sheets (43%) and relatively poor in α-helices (30%), detergent insoluble and usually partially resistant to proteolysis.³ The detailed mechanism of PrPC-to-PrPSc conversion remains unclear, but PrPC is required not only for PrPSc replication but also for prion pathogenesis.

The normal PrPC functions are still elusive, but PrPC has been implicated in many physiological and pathological processes. An early report implicates PrPαC in lymphocyte activation.⁴ Depletion of PrPC is relatively innocuous: the PrP-null mice live a normal lifespan without displaying obvious developmental defects and only present subtle phenotypes, such as mild cognitive and behavioral deficits.⁵ Postnatal knockout of PrP expression in the brain did not affect neuronal survival in transgenic mice either.⁶ In neurons, PrPC is mainly localized at synapses, in cholesterol-rich micro-domains,⁷,⁸ PrPC was reported to play a functional role in neuronal cell adhesion, migration and differentiation by modulating different cell-signaling pathways⁹ and it interacts with several neuronal proteins, including Bcl2, Bax, stress-inducible protein 1, as well as with cell adhesion molecules or extracellular matrix proteins, such as laminin, vitronectin and NCAM, to mediate the neuritogenesis and neuronal differentiation in several cell models.¹⁰-¹⁵ Besides, PrPC was shown to be neuroprotective through influencing neuronal and glial factor involved in antioxidative defense in a mouse model of amyotrophic lateral sclerosis¹⁶ but to enhance stauroporine-induced apoptosis via regulating p53 in cultured neuronal cells.¹⁷,¹⁸ PrPC also seems to play important roles in other tissues/cells: it is important for the self-renewal of long-term repopulating hematopoietic stem cells,¹⁹ recruits important interacting signaling molecules to influence T cells activation through association with the lipid raft proteins reggie-1 and reggie-2,²⁰ and functions in embryogenesis by regulating embryonic cell adhesion.²¹-²³ Moreover, PrPC has been reported to regulate Aβ production²⁴-²⁶ and serve as the receptor for the cytotoxic Aβ oligomers²⁷ although contradicting findings were reported.²⁸,²⁹

Three Types of PrP Processing

Like many other cell surface proteins such as amyloid precursor protein, PrPC can be differentially cleaved at specific sites to generate various fragments (Fig. 1, Tables 1–2). A secreted form of full length PrPC was first observed in 1993 in both the medium of cultured cells and the human cerebrospinal fluid.³⁰,³¹
Several subsequent reports suggest that such shedding occurs near the site of GPI anchor and result from a secretase-like proteolytic cleavage by zinc metalloproteases of the ADAM family.32-35 In 2009, the Hooper group reported that ADAM10 but not ADAM17 cleaves PrP\(^\text{C}\) between Gly\(^{228}\) and Arg\(^{229}\) as measured by N-terminal sequencing.40 This cleavage was termed \(\alpha\)-cleavage,32 and the resulting C-terminal product is named C1,31-33,39,40,45,51 This review will focus on the better studied and functionally significant \(\alpha\)-cleavage.

\[\text{\(\alpha\)-Cleavage of PrP}\(^\text{C}\)]

In 1993, Harris et al. first reported that chicken PrP\(^\text{C}\) undergoes a cleavage in the central hydrophobic domain within a region of 24 amino acids, representing a major processing event that may have physiological as well as pathological significance.31 In 1995, Chen et al. detected abundant similar PrP cleavage products in human brains and human neuroblastoma cells and established that the precise cleavage site is between residues 110/111 or 111/112 as measured by N-terminal sequencing.49 This cleavage was termed \(\alpha\)-cleavage,32 and the resulting C-terminal product is named C1, which was found to be mostly associated with the membrane fraction in a glycosylated and GPI-anchored form on the cell surface.40 but it is heat-stable and more acidic.31,40 The corresponding N-terminal fragment (termed N1) was subsequently detected in cell culture medium and cerebral spinal fluid.33,48,51,52

Significance of PrP\(^\text{C}\) \(\alpha\)-cleavage. The PrP109-122 region is one of the \(\alpha\)-helical regions that was postulated to acquire a \(\beta\)-sheet
structure in PrPSc and it plays a critical role in the conformational changes underlying the conversion of PrPc to PrPSc.53-58 PrP106-126 peptide is prone to form fibrils similar to those present in the PrP amyloid plaques of prion diseases,59 and it is cytotoxic.60-62 The α-cleavage between PrP amino acid residues 110-111 or 111-112 disrupts the PrP106-126 region that is critical for both prion replication and PrP toxicity and generates the bioactive N1 and C1 fragments. The Checler group found that the N1 fragment is neuroprotective by reducing p53-dependent cell death both in vitro and in vivo,63 and it protects against monomeric and oligomeric Aβ toxicity in culture cells.64 The functions of C1 are debatable. The Checler group showed that the C1 fragment enhanced the apoptotic effect of staurosporine through positively regulating caspase-3 activation in a p53-dependent mechanism in the presence of PrPc in HEK293 cells, but overexpression of C2 fragment appeared to have no effect.67 In contrast, data from the Harris group suggest that C1 is non-toxic and protective against prion infection.64 They generated the Tg(C1) transgenic mice expressing PrP(Δ23-111) that corresponds to C1,66 and found that in the absence of endogenous PrP, Tg(C1) mice displayed no signs of neurological symptoms or histological lesions even when C1 was expressed at 7 times of normal PrP levels, indicating that C1 is not neurotoxic; Tg(C1) mice with one or both copies of the endogenous Prnp gene were also clinically and neurohistologically normal. The apparent discrepancy is likely due to the lack of treatment with pro-apoptotic agent in the in vivo study while the apoptosis-enhancing effect of C1 in the in vitro cell assays was detected only under staurosporine treatment. Therefore, it appears that C1 only enhances susceptibility to pro-apoptotic stimuli (such as staurosporine) but it is not neurotoxic under normal conditions. In addition, in the absence of endogenous PrP the Tg(C1) mice inoculated with scrapie prions remained healthy and did not accumulate protease-resistant PrP, indicating that C1 is not a substrate for conversation to PrPSc. Moreover, in scrapie-inoculated mice expressing wild type mouse PrP, co-expression of C1 led to a dramatically delayed time course and markedly slowed PrPSc accumulation, demonstrating that C1 is a dominant-negative inhibitor of PrPSc accumulation and prion disease progression.64

**Subcellular site of PrPc α-cleavage.** The precise subcellular location for α-cleavage remains controversial. The Harris group reported in 1993 that chicken PrPc was proteolytically cleaved within a highly conserved region in the NH₂-terminal half of the molecule and this cleavage was reduced by lysosomotropic amines and inhibitors of lysosomal proteases, suggesting that it occurs in an acidic endocytic compartment.65 However, the Hooper group reached different conclusions.65 Utilizing a human neuroblastoma cell line (SH-SY5Y), they found that C1 was detected at the cell surface and its production was not dependent on Cu²⁺-mediated PrP endocytosis; the GPI anchor is also not required either since a transmembrane-anchored form that is not associated with the lipid raft and a secreted construct lacking the GPI membrane anchor were still subject to α-cleavage, but a transmembrane-form containing an endoplasmic reticulum retention motif failed to produce C1 and inhibition of protein export from the Golgi by temperature block led to elevated C1. These data strongly argue for a late compartment of the secretory pathway as the site for PrPc α-cleavage.65

**Regulation of PrPc α-cleavage.** The Checler group reported that production of secreted N1 fragment was increased by the protein kinase C agonists PDMu and PMA (both phorbol esters) in a time- and dose-dependent manner in mouse TSM1 neurons and human HEK293 cell, but the protein kinase A effectors dibutyryl cAMP and forskolin had no effect,52 indicating that the normal processing of PrPc (at least the secreted N1 level) is upregulated by protein kinase C but not protein kinase A. The same group later presented evidence from mouse embryonic primary neurons and HEK293 cells to show that the M1 and M3

| Type of processing | Factors involved | References | Results |
|--------------------|------------------|------------|---------|
| β-cleavage         | Reactive oxygen species | McMahon et al. (2001) | β-cleavage of PrPc by reactive oxygen species is copper- and pH-dependent. |
|                    | Calpain          | Watt et al. (2005) | ROS-mediated β-cleavage of PrPc is an early and critical event associated with protection against oxidative stress |
|                    | Cathepsin        | Dron et al. (2010) | Cathepsin but not calpain inhibitors inhibited C2 formation, indicating that acidic hydrolases of the endolysosomal compartment is involved in the β-cleavage of PrPSc that is cell- and tissue-dependent. The N-termini of in vivo and in vitro generated C2 differ. |
| Shedding near the GPI anchor | ADAM10 | Taylor et al. (2009) | ADAM10, but not ADAM17, are involved in the ectodomain shedding of PrPc. ADAM10 directly cleaves murine PrP between Gly228 and Arg229. |
|                    | ADAM9            | Altmmeppen et al. (2011) | ADAM10 is the sheddase of PrPc in vivo and the lack of ADAM10 leads to increased amounts and accumulation of PrPc in the early secretory pathway by affecting its posttranslational processing. |
| Shedding at the GPI anchor | Phospholipase | Parkin et al. (2004) | Lipid raft-disrupting agent-mediated shedding of PrPc is likely to occur via phospholipase cleavage of the GPI anchor. |
muscarnic receptors regulate N1 production by modulating the phosphorylation state and activity of ADAM17. A follow-up report revealed that the ERK1 kinase regulates both N1 secretion and PrP mRNA levels. 

**Proteases responsible for the α-cleavage of PrP**. ADAM10, ADAM17, and ADAM9. There have been conflicting reports on the proteases responsible for the α-cleavage of PrP. The Checler group reported that, in human HEK293 cells, α-phenanthroline (a general zinc-metalloprotease inhibitor), BB3103 (inhibitor of metalloprotease ADAM10) and TAPI (inhibitor of tumor necrosis factor α-converting enzyme (TACE or ADAM17)) treatment dramatically reduced N1 levels. In HEK293 cells treated with phorbol 12,13-dibutyrate (PDBu), when compared with untreated and untransfected HEK293 cells, overexpression of human TACE resulted in a > 2-fold increase in N1 levels while overexpression of human ADAM10 led to a ~30% increase in N1 level; however, the N1 levels in HEK293 cells overexpressing ADAM10 or TACE in the absence of PDBu treatment were not reported. In mouse embryonic fibroblasts, when the ADAM10 gene was knocked out, the constitutive N1 secretion was reduced by half in ADAM10-null cells and PDBu-regulated N1 production remained; in contrast, in ADAM17-deficient cells, PDBu treatment had no effect on N1 level, and the constitutive N1 level was unchanged compared with the wild type cells. Assuming that N1 production and its regulation is the same in HEK293 cells and mouse embryonic fibroblasts, these data support the conclusion that ADAM10 is involved in constitutive N1 production while TACE (ADAM17) mostly contributes to phorbol ester regulated N1 formation. It is worth noting that the N1 level was reduced by only about half in ADAM10-null embryonic fibroblast cells, there must be other protease(s) involved in constitutive N1 production. These data argue that ADAM10 and ADAM17 are candidates for the α-cleavage of PrP. In a follow-up report, the Checler group showed that decreasing endogenous ADAM9 expression by an anti-sense approach dramatically reduced N1 secretion, and transient transfection with ADAM9 cDNA alone failed to increase N1 production in ADAM10-null fibroblasts while co-expression of ADAM9 and ADAM10 in ADAM10-null fibroblasts led to more N1 than transfection with ADAM10 cDNA alone, suggesting that ADAM9 indirectly contributes to the production of N1 via ADAM10. However, these pioneering studies have a couple of caveats. First, only secreted N1 in the serum-deprived condition medium was measured. N1 has been detected inside cells and it has been suggested that the α-cleavage of PrP likely mostly occurs in an intracellular compartment. Further examination of intracellular N1 would give a more complete picture. Second, C1 levels were not measured except for the experiment showing that carbobol treatment increased C1 levels in HEK293 cells overexpressing M1-muscarinic receptor. Third, evidence for direct α-cleavage of PrP by purified ADAM10 or ADAM17 proteins is still lacking. Indeed, when the Hooper group examined the C1 level in HEK293 cells, they found that overexpression of ADAM9, ADAM10 and ADAM17 by transfection of corresponding cDNA and knocking-down of ADAM9 and ADAM10 by siRNA did not have significant effect on the PrP C1/full-length ratio. Moreover, in vitro cleavage assay with recombinant PrP and recombinant ADAM10 and mass spectrometric analysis of the reaction products did not show any prominent peaks other than those representing a near GPI-anchor shedding product; however, there was no discussion on whether C1 was detectable at low levels. Laffont-Proust et al. found big inter-individual variation of PrP C1/full length ratio in human cerebral cortex and high levels of C1 seem to be associated with the presence of active ADAM10 protein although the protein levels of other ADAMs were not examined. However, Endres et al. reported that, instead of increasing the C1/full-length ratio, neuronal overexpression of ADAM10 in transgenic mice significantly reduced all PrP species probably through interfering with PrP gene expression. In addition, recombinant human ADAM10 and ADAM17 both failed to cleave a 17-residue human PrP peptide substrate containing the α-cleavage site. In contrast, assays by the Checler group using intact cells and a fluorimetric substrate (JMV2770) mimicking the α-cleavage site demonstrated that overexpressing ADAM10 leads to cleavage of the substrate, ADAM9 is indirectly involved likely via shedding of ADAM10, and the apparent α-cleavage activity of ADAM17 is upregulated by its phosphorylation at Thr-735. Moreover, Altmeppen et al. found that neuron-specific knockout of ADAM10 in transgenic mice led to increased PrP levels in the brain and primary neurons through a post-translational mechanism as well as accumulation of PrP in the early secretory pathway, but it had no effect on the α-cleavage of PrP albeit it did drastically reduce the shedding of the PrP ectodomain. These conflicting results raise questions on the exact roles of ADAM10, ADAM9, and ADAM17 in the α-cleavage of PrP. One possibility is that a yet-to-be identified protease(s) directly performs the α-cleavage and ADAM10 and ADAM17 indirectly affect N1 production through modulating this other protease(s), but it is also possible that only certain post-translationally modified ADAM10 and/or ADAM17 protein can directly perform α-cleavage of PrP.

**Plasmin and plasminogen.** Plasmin is processed from its precursor plasminogen. As a serine protease responsible for thrombosis, plasmin participates in the extravascular breakdown of matrix and basement membrane glycoproteins. Plasminogen is abundantly expressed from the liver, but it is also broadly expressed in extrahepatic tissues, including adrenal gland, kidney, heart, muscle, lung, uterus, testis, spleen, thymus and brain (including neurons and microglia). In 2003, Kornblatt et al. reported that plasminogen purified from human plasma could form a complex with recombinant full-length sheep PrP and cleave it to generate the C1 fragment in vitro as confirmed by mass spectrometric analysis, and this cleavage was accelerated but not dependent on the presence of plasmin. Praus et al. showed that the N1 fragment together with low molecular weight heparin stimulated t-PA mediated plasminogen activation in vitro, suggesting that N1 could potentially augment PrP α-cleavage through enhanced plasminogen activation to produce more N1. However, in a subsequent paper, the Kretzschmar group found that the cleavage pattern of PrP in brain and other tissues of plasminogen-deficient mice was unchanged from that of wild-type mice, suggesting that plasmin may not perform PrP α-cleavage in vivo. The reason for
the discrepancy between in vitro and in vivo data is unknown. Given that plasminogen is also expressed in many tissues with PrP expression, it would be worthwhile to revisit the roles of plasmin and plasminogen in PrP\(^\alpha\)-\(\alpha\)-cleavage.

**ADAM8**. We have studied the \(\alpha\)-cleavage of PrP\(^\alpha\) in skeletal muscles by examining the C1 levels and PrP C1/full length ratios.\(^7\) The PrP C1/full-length ratio is a more reliable indicator of \(\alpha\)-cleavage activity because the PrP\(^\alpha\) level itself would influence the C1 and N1 levels. We established a transgenic mouse model named Tg(HQK) that shows strictly doxycycline (Dox)-dependent muscle-specific expression of wild type human PrP.\(^7\) We found that in the skeletal muscles of Dox-induced Tg(HQK) mice, starting from day 5 of Dox induction, the C1 levels and C1/full length ratio started rising dramatically, with the ratio reached a peak of \(-3.0\) at day 7 and remained there afterwards. Quantitative real-time PCR analysis of the mRNAs for six ADAMs (ADAM8, ADAM9, ADAM10, ADAM12, ADAM17, and ADAM23) revealed that only ADAM8 mRNA level rose significantly in the muscles of Dox-treated Tg(HQK) mice.\(^7\) Western blot analysis showed that ADAM8 protein level started to rise at day 4 of Dox treatment, which preceded the dramatic rise of C1/full length ratio, and it kept rising slowly with continued Dox treatment. Examination of skeletal muscle tissues from four transgenic mouse lines constitutively expressing wild type human PrP at different levels revealed that the PrP C1/full length ratio was linearly correlated with the ADAM8 protein level. Such a linear correlation was also found in PrP-expressing C2C12 myoblast cell lines where ADAM8 expression was knocked down to different levels by siRNA. In addition, the C1/full length ratio is decreased dramatically in the skeletal muscles of ADAM8-null mice. Moreover, we demonstrated that recombinant human ADAM8 protein could directly cleave recombinant human PrP to generate C1 fragment. The in vitro production of C1 fragment was detected by direct protein staining with Commassie blue, immunoblotting with PrP antibodies and mass spectrometric analysis; in vitro production of the N1 fragment was also confirmed by mass spectrometry.\(^7\) Our results demonstrate that ADAM8 is the primary protease responsible for direct \(\alpha\)-cleavage of PrP\(^\alpha\) in muscle cells. However there must be another unidentified protease(s) contributing to the \(\alpha\)-cleavage of PrP\(^\alpha\) in muscle cells given the residual C1 in ADAM8-KO muscle tissues. Whether this other protease(s) is elevated ADAM8 activity and enhanced PrP \(\alpha\)-cleavage in the PrP-overexpressing muscles will produce more N1 fragment that may partially protect against the toxic effects of overexpressed PrP\(^\alpha\).

The role of ADAM8 in the \(\alpha\)-cleavage of PrP\(^\alpha\) in neuronal cells, brain and other tissues is under examination. Our preliminary result seems to suggest a less prominent role for ADAM8 in the \(\alpha\)-cleavage of PrP\(^\alpha\) in the brain (Li and Kong, unpublished), pointing to the possibility of tissue- and cell type-dependent \(\alpha\)-cleavage of PrP\(^\alpha\). We also found that overexpression of PrP\(^\alpha\) in the muscle tissues or C2C12 myoblast cells leads to elevated ADAM8 protein level, suggesting a feedback loop in muscle cells where PrP\(^\alpha\) modulates its own \(\alpha\)-cleavage through regulating ADAM8 expression, likely at the transcription level.\(^7\) ADAM8 is mainly expressed in the immune cells, such as monocytes, neutrophils, eosinophils, dendritic cells, and B cells, and in muscles and brain (Li and Kong, unpublished data), and it is known to play an important role in allergic respiratory diseases (such as asthma) and cancer.\(^8\) PrP\(^\alpha\)-mediated regulation of ADAM8 may suggest a potential novel mechanism for PrP\(^\alpha\) in the allergic respiratory diseases and cancer via its complex relationship with ADAM8.

**Summary**

The \(\alpha\)-cleavage of PrP\(^\alpha\) produces functionally significant N1 and C1 fragments and disrupts a region critical for both prion replication and PrP toxicity. ADAM8, ADAM10, ADAM17, and ADAM9 are directly or indirectly involved in the \(\alpha\)-cleavage of PrP\(^\alpha\), but more research is needed to fully understand all participating enzymes as well as their tissue-specificity and regulation. The relationships between PrP\(^\alpha\) and its processing enzymes also deserve close scrutiny as it may reveal novel roles for PrP\(^\alpha\) in various biological processes and non-prion diseases.

**Disclosure of Potential Conflicts of Interest**

The authors declare no conflicts of interest.

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