A New Paradigm for Enzymatic Control of α-Cleavage and β-Cleavage of the Prion Protein*

Alex J. McDonald, Jessie P. Dibble, Eric G. B. Evans, and Glenn L. Millhauser

From the Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

Background: The prion protein (PrP) is cleaved into bioactive fragments by enzymes and metal catalysis.

Results: Key members of the ADAM protease family yield all observed PrP products.

Conclusion: Proteases alone may be responsible for PrP processing, with distinct activities depending on specific enzymes.

Significance: Elucidation of the responsible enzymes and products can be used to evaluate PrP function and proteolysis in prion disease.

The cellular form of the prion protein (PrPc) is found in both full-length and several different cleaved forms in vivo. Although the precise functions of the PrPc proteolytic products are not known, cleavage between the unstructured N-terminal domain and the structured C-terminal domain at Lys-109 ↓ His-110 (mouse sequence), termed α-cleavage, has been shown to produce the anti-apoptotic N1 and the scrapie-resistant C1 peptide fragments. β-Cleavage, residing adjacent to the octarepeat domain and N-terminal to the α-cleavage site, is thought to arise from the action of reactive oxygen species produced from redox cycling of coordinated copper. We sought to elucidate the role of key members of the ADAM (a disintegrin and metalloprotease) enzyme family, as well as Cu2+-dependent manner. Additional cleavage by ADAM8 was observed at the previously proposed location of α-cleavage, Lys-109 ↓ His-110 (MoPrP sequencing); however, upon addition of Cu2+, the location of α-cleavage shifted by several amino acids toward the C terminus. ADAM10 and ADAM17 have also been implicated in α-cleavage at Lys-109 ↓ His-110; however, we observed that they instead cleaved MoPrP at a novel location, Ala-119 ↓ Val-120, with additional cleavage by ADAM10 at Gly-227 ↓ Arg-228 near the C terminus. Together, our results show that MoPrP cleavage is far more complex than previously thought and suggest a mechanism by which PrPc fragmentation responds to Cu2+ and Zn2+.

The prodomain (PrPd) is an abundant extracellular glycosylphosphatidylinositol (GPI)-anchored glycoprotein consisting of a helical C-terminal domain and a partially structured N-terminal domain (Fig. 1) (1, 2). Prion diseases, which result from conversion of cellular PrP (PrPc) to the misfolded aggregated scrapie form (PrPSc), include Creutzfeldt-Jakob disease and kuru in humans, bovine spongiform encephalopathy, chronic wasting disease in deer and elk, and scrapie in goats and sheep (3, 4). The full-length protein is ~210 amino acids long, but PrPc is also found in several distinct truncated forms in vivo, with proteolytic species possessing unique bioactive properties (5, 6).

In healthy tissues, enzyme cleavage of PrPc at approximately Lys-109 ↓ His-110 (mouse sequence), termed α-cleavage, produces the N- and C-terminal fragments, N1 and C1, respectively. The preponderance of recent evidence suggests that α-cleavage, which separates most of the PrP N terminus from the folded C terminus, is due to action from one or more members of the ADAM (a disintegrin and metalloprotease) family of enzymes, specifically ADAM8, ADAM10, and ADAM17 (7, 8). α-Cleavage was originally assigned to ADAM10, but recent studies found that neither knocking out ADAM10 nor treatment with ADAM10-specific protease inhibitors is capable of blocking α-cleavage and instead blocks proteolysis of PrPc near the C-terminal GPI anchor at Gly-227 ↓ Arg-228 (9 –11). More recently, ADAM8 was identified as the protease responsible for α-cleavage in skeletal muscle tissue (7). Interestingly, a study aimed at identifying the specific α-cleavage site found that PrPc exhibits remarkable tolerance to point mutations and deletions of short segments (12). Deletion of the extended segment 100–129 was necessary to fully block cleavage.

The prodromas released by α-cleavage exhibit potent activities. The N1 fragment is anti-apoptotic, possibly acting through the inhibition of caspase-3 (13). Conversely, the C1 fragment promotes apoptosis through p53-dependent caspase-3 activity, although it appears as though the protective effects of N1 significantly outweigh the pro-apoptotic effects of C1 (14). Perhaps more importantly, substoichiometric levels of C1 protect against PrPSc propagation (15, 16).

In Creutzfeldt-Jakob disease patients, postmortem brain homogenates present a second cleavage site, termed β-cleavage, which produces N2 and C2 fragments (6). Because the C2 fragment from the homogenates is proteinase K-resistant and migrates the same distance upon gel electrophoresis as the proteinase K-digested PrPSc core (proteinase K-resistant PrP), it was originally proposed that the observed C2 fragment from...
Creutzfeldt-Jakob disease brain tissue corresponded to proteinase K-resistant PrP, placing β-cleavage at Gly-89 → Gln-90. Subsequently, a soluble cleavage fragment of PrP[^C] determined by antibody staining to be similar to C2 was observed in scrapie-free CHO and SH-SYSY cell lines expressing PrP[^C] (17, 18). The observed levels of C2 are greatly enhanced upon addition of H_2O_2, suggesting proteolysis by reactive oxygen species (ROS) generated by intrinsic copper, which binds with high affinity to the N-terminal octarepeats (residues 60–91, human sequence). However, there have been several recent studies implicating proteases from the calpain and cathepsin families in β-cleavage of PrP[^Sc], leaving open the possibility that β-cleavage in PrP[^C] could also be enzymatic (19, 20). Unlike the N1 and C1 fragments, N2 and C2 do not show any bioactivity or neuroprotection, although β-cleavage production of N2 and C2 may indirectly assert a biological effect by denying the formation of N1 and C1 (13–15).

PrP[^C] is enzymatically shed from its GPI membrane attachment by the action of ADAM10 (10). Free PrP[^C] is found in cell culture and human cerebral spinal fluid. Recent literature suggests several possible functions for PrP[^C], distinct from its N1 and C1 fragments. For example, full-length PrP[^C] may trap pro-amyloid-β (Aβ) oligomers, implicated in Alzheimer disease, in its hydrophobic segment, thereby acting in defense against neurodegenerative disease (21). In addition, membrane-bound PrP[^C] stimulates NMDA receptors (22), an activity that may be neuroprotective (23). In addition, membrane-bound PrP[^C] stimulates NMDA receptors (22), an activity that may be neuroprotective (23). However, PrP[^C] cleavage results from aberrant copper redox activity and is associated with the development of prion disease. Recent work from our laboratory (24) identified a metal ion-driven tertiary structure between the PrP[^C] N- and C-terminal domains, thereby suggesting a re-evaluation of the relative roles of ADAM proteases and copper and zinc in proteolysis. Using Zn^{2+}, which is both a natural PrP[^C]-binding metal and a surrogate for Cu^{2+}, Spevacek et al. (24) found that the metal-occupied octarepeat domain makes contact with the C-terminal surface formed by helices 2 and 3. This interaction is systemically weakened by familial mutations within these two helices. The compact protein resulting from this newly identified contact likely restricts conformational freedom of the proteolytically susceptible linker (residues 91–125), which encompasses the α-cleavage site.

In this work, we use LC/MS to examine the relative activities of ADAM8, ADAM10, and ADAM17 on recombinant mouse PrP[^C] (MoPrP[^C]) in the absence and presence of both Zn^{2+} and Cu^{2+}. We show that Cu^{2+}-generated ROS, ADAM8, ADAM10, and ADAM17 are all capable of cleaving MoPrP, albeit at distinct locations. Surprisingly, we observed that in the absence of ROS, ADAM8 cleaved MoPrP[^C] at both α- and β-cleavage locations in a Cu^{2+}- and Zn^{2+}-dependent manner. Moreover, we found that α-cleavage occurred at several nearby locations depending on the specific enzyme and condition. Through our assay, we were able to design mutants that block certain cleavage events by ADAM8, ADAM10, and ADAM17, an advance that could prove useful in further analysis of the role of enzymes in PrP[^C] function and prion disease. Finally, we observed that the familial mutants show altered α-cleavage profiles. As opposed to metal-catalyzed backbone scission, our findings suggest a new paradigm whereby physiologic metal ions regulate enzyme-driven PrP[^C] processing, controlling the relative α- and β-cleavage products.

**EXPERIMENTAL PROCEDURES**

**Protein Expression**—The prion protein was expressed and purified as described previously (25). In brief, MoPrP[^C] (23–230) was cloned into the pET101 vector using the Champion™ pET101 Direction TOPO® expression kit (Invitrogen). Mutant constructs were made with the pET101-MoPrP[^C] (23–230) plasmid as the template using the GeneArt® site-directed mutagenesis kit (Invitrogen). All constructs were verified by DNA sequencing. The pET101 vector was then transformed into BL21 Star™ cells and expressed for 20 h via autoinduction (26). Cells were lysed, and inclusion bodies containing MoPrP were purified from the lysate. All constructs were then purified with an ÄKTA purifier (GE Healthcare) using a Ni^2+ immobilized metal ion affinity column with the exception of MoPrP (90–230), which was purified using a Q-Sepharose anion exchange column. Protein was eluted from the Ni^{2+} immobilized metal ion affinity column with 8 M guanidine HCl (pH 4.5) while monitoring A_{280}. Fractions spanning the elution peak were combined, and the pH was raised to 8, followed by storage at 4°C for 2 days to facilitate proper folding. The protein was then purified by reverse-phase HPLC using a C_{4} column (Grace/Vydac), and the correct mass of the purified protein was verified by electrospray ionization mass spectrometry. Fractions containing the purified protein were pooled, lyophilized, and stored at −20°C for future use. Protein stocks were reconstituted in 0.2-μm filtered water and quantified with a NanoDrop UV-visible spectrophotometer (Thermo Scientific) prior to use.
Cleavage of MoPrP by ROS—Cleavage of MoPrP was carried out by forming ROS in vitro using ascorbic acid and Cu²⁺. 50-µl samples were made containing 100 μM MoPrP, 25 mM MOPS (pH 7.4), and 1 mM ascorbic acid. To begin the reaction, Cu(OAc)₂ was added to a final concentration of 200 μM. For control samples, Cu²⁺ was omitted from the reaction, resulting in no cleavage. Although the above conditions produced cleavage of MoPrP within the octarepeats, it was difficult to identify peaks by mass spectroscopy due to excessive oxidation from H₂O₂ generated by Cu²⁺/ascorbic acid redox cycling. To alleviate excessive oxidation, the H₂O₂ decomposition enzyme catalase (Spectrum) was added to the reaction to a final concentration of 0.1 mg/ml. Samples were allowed to incubate overnight at 25 °C. Once the reaction was completed, it was quenched using the free radical scavenger dimethyl sulfoxide, which was added to a final concentration of 1%.

ADAM8 Cleavage of MoPrP—Recombinant human ADAM8 was purchased from R&D Systems. ADAM8 must be activated before use by cleaving a propeptide with thermolysin. First, a fresh stock of 1.5 μg/ml thermolysin was made with ADAM8 primary buffer (50 mM Tris, 10 mM CaCl₂, and 150 mM NaCl (pH 7.5)), and separately, a 0.5 mM stock of the thermolysin inhibitor phosphoramidon (Santa Cruz Biotechnology) was made with ADAM8 primary buffer. Recombinant ADAM8 was diluted to 400 μg/ml in ADAM8 primary buffer, and then an equal volume of 1.5 μg/ml thermolysin stock was added. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of phosphoramidon to a final concentration of 0.05 mM and incubated at 25 °C for 15 min. Thorough controls were performed to ensure that this process fully inactivated thermolysin (data not shown). Before use, the activated ADAM8 was diluted 5-fold with dilution buffer (20 mM Tris, 5 mM CaCl₂, and 25 mM KCl (pH 7.4)). Separately, working stocks of 40 μM MoPrP constructs were made from lyophilized protein brought up in dilution buffer and quantified by UV-visible spectroscopy. For reactions testing the effects of Cu²⁺ or Zn²⁺ on ADAM8 cleavage, the appropriate molar equivalents of Cu(OAc)₂ or ZnCl₂, were added to the 40 μM MoPrP solutions, followed by incubation at 25 °C for 15 min. It is important to use the low salt dilution buffer, or MoPrP will precipitate out of solution in the presence of Cu²⁺ or Zn²⁺. 15 μl of dilute activated ADAM8 and 15 μl of MoPrP construct were combined and allowed to react for 6 h at 37 °C unless noted otherwise. The reaction was stopped by the addition of 5 μl of 1% formic acid and stored at 4 °C until assayed. For the time trial of ADAM8 activity on MoPrP, the reaction was quenched at each time point by the addition of the metalloproteinase inhibitor Batimastat to a final concentration of 5 μM and then stored at 4 °C until assayed.

ADAM10 and ADAM17 Cleavage of MoPrP—Recombinant human ADAM10 and ADAM17 were purchased from R&D Systems. ADAM10 was diluted to 2 ng/µl in ADAM10 assay buffer (25 mM Tris, 2.5 μM ZnCl₂, and 0.005% Brij-35 (pH 7.4)), and ADAM17 was diluted to 0.5 ng/µl in ADAM10 assay buffer. MoPrP constructs were diluted to 40 μM in ADAM10 assay buffer. 15 μl of diluted ADAM10 or ADAM17 was combined with 15 μl of MoPrP construct and incubated overnight at 37 °C. Cleavage reactions were stopped by the addition of 1 μl of 1% formic acid and stored at 4 °C until assayed.

LC/MS—MoPrP cleavage samples were spun down for 5 min at 13,000 relative centrifugal force to pellet any debris and material that may have precipitated during sample preparation. 30 μl of supernatant was then pipetted into autosampler vials and loaded into an LTQ orbitrap LC/MS autosampler (Fisher), chilled to 4 °C. 20 μl of cleavage products was drawn from a vial and separated with a C₄ HPLC column (Higgins Analytical) using a 60-min gradient of water/acetonitrile mobile phases. The A₂₈₀ was continuously recorded by a photodiode array, whereas mass spectra were continuously taken using an LTQ orbitrap mass spectrometer (Fisher). The C₄ column was flushed with 95% acetonitrile to remove any residually bound protein and then re-equilibrated with 95% water between each sample run.

Cleavage Product Identification and Analysis—The LC/MS spectra from each sample run were first analyzed by MS BioWorks. The mass spectrum ladder for each peak separated by the C₄ column was deconvoluted using BioWorks to reveal the parent mass of the cleavage product (data not shown). The masses of the observed peaks were cross-referenced against the predicted masses of hydrolysis of all possible peptide bonds of MoPrP to determine which potential cleavage fragment each observed peak corresponded to. For all cleavage fragments enzymatically produced, observed masses were within 1 atomic mass unit of the mass of a predicted cleavage fragment. Fragments produced by ROS tended to show one or more oxidations. Once peaks were annotated with the cleavage fragment that produced them, the A₂₈₀ of each peak was separately integrated in Xcalibur (Thermo Scientific), ensuring that integrations spanned the retention time range corresponding to the proper fragment mass. Peak integrals were then divided by the extinction coefficient of their corresponding fragment to give relative abundance. Error bars shown for the Cu²⁺ titration of ADAM-PrP cleavage (n = 3) are representative of the reproducibility of LC/MS cleavage assays, typically within 10% error (see Fig. 4, A and B).

RESULTS

ROS Generated from Cu²⁺ Redox Cycling Are Capable of Cleaving MoPrP within the Octarepeat Domain—In vivo work has established that Cu²⁺-generated ROS are a mechanism underlying β-cleavage of PrP⁰C (17); however, the location of the cleavage site has only been approximated by Western blot analysis (6). To further understand the physiological events leading to N2 and C2 production, we sought to first determine the precise location of ROS-driven β-cleavage in vitro. Copper and the naturally occurring reductant ascorbic acid produce hydroxyl radicals (HO·), a common component of ROS. Copper acetate and ascorbic acid at concentrations of 200 μM and 100 mM, respectively, were added to a solution of 100 μM MoPrP and reacted overnight. To avoid spurious oxidation events at amino acid side chains, we also included catalase, which rapidly inactivates excess H₂O₂ but not HO·. As shown in Fig. 2, LC/MS revealed a clear C2 fragment, along with multiple N-terminal peptides. Evaluation of the N2 products showed that MoPrP was cleaved following each of the four histidines

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FIGURE 2. Redox cycling of Cu²⁺ coordinated to MoPrP is capable of producing β-cleavage fragments. 100 μM MoPrP was incubated with 2 eq of Cu²⁺ in the absence or presence of 100 mM ascorbic acid (AA) overnight and then assayed by LC/MS. The resulting redox cycling of Cu²⁺ produced ROS capable of cleaving MoPrP following the four histidines of the N-terminal octarepeat domain (His-60 ↓ Gly-61, His-68 ↓ Gly-69, His-76 ↓ Gly-77, and His-84 ↓ Gly-85). A, peaks corresponding to the N- and C-terminal fragments were identified in the LC trace of MoPrP with 2 eq of Cu²⁺ and 100 mM ascorbic acid (dashed line) but were not seen in the absence of ascorbic acid (solid line). B, significant peaks from the LC trace were identified by mass spectrometry and then integrated to determine the relative abundance of the peptide fragments. The four N-terminal fragments produced from cleavage at each histidine in the octarepeats co-eluted and were integrated together as N2. Likewise, the four complementary C-terminal fragments co-eluted as a distinct shoulder of the peak for full-length protein and were integrated together as C2.

MoPrP Is Cleaved by ADAM8 in Vitro in a Cu²⁺-dependent Fashion—ADAM8 produces α-cleavage of MoPrP in vitro (7). To identify the specific cleavage point and to evaluate other possible products, MoPrP(C) was treated with ADAM8, but without additional reagents that might produce ROS. MoPrP (20 μM) was incubated with 20 ng/μl activated recombinant ADAM8 for 6 h in a low salt pH 7.4 buffer, conditions that minimize MoPrP precipitation (Fig. 3A). The resulting cleavage pattern was determined by mass analysis of the individual fragment peaks, followed by correlation of the peptide masses to the cleavage locations in MoPrP. Fragments corresponding to cleavage of MoPrP at the expected site of α-cleavage, Lys-109 ↓ His-110, were observed to within 1 Da of the predicted masses of N1 and C1; this event will henceforth be referred to as α₁-cleavage. Surprisingly, additional fragments were observed corresponding to β-cleavage in the octarepeat domain at Pro-59 ↓ His-60, Pro-67 ↓ His-68, Pro-75 ↓ His-76, and Pro-83 ↓ His-84, as well as a small amount of additional α-cleavage centered on Ala-116 ↓ Ala-117. This latter proteolysis event will be referred to as α₂-cleavage and is somewhat ragged with variation of the cut point by two or three Ala residues. It is interesting to note that Cu²⁺-mediated ROS produced β-cleavage following each histidine, whereas ADAM8 β-cleavage took place before each histidine. Additional smaller unannotated peaks, which resulted from proteolysis at the two cleavage sites, indicate that both α- and β-cleavage can occur within the same protein molecule.

A primary location for copper binding in PrP(C) is in the octarepeat domain, composed of four tandem repeats of the fundamental eight-residue segment PHGGGWGQ (27). Within this domain, Cu²⁺ is taken up with two distinct coordination modes depending on the copper concentrations. At low copper levels, a single Cu²⁺ ion coordinates to the four octarepeat histidines with a K_d of ~0.1 nM. At high copper levels, up to four Cu²⁺ ions are taken up, with one metal ion in each of the repeating PHGGGWGQ sequences (coordinated specifically to the HGGGW subsequence). The affinity is significantly lower, with a K_d of ~10 μM (28). An additional 2 eq of Cu²⁺ are coordinated outside the octarepeat domain by His-95 and His-110 (mouse sequence) with a K_d of ~0.1 nm (29). Alternatively, the octarepeat domain may take up a single Zn²⁺ ion through the four histidines with an affinity of ~200 μM (29).

Given that copper coordinates to PrP(C) in segments that are susceptible to proteolysis, we examined the effects of Cu²⁺ on ADAM8 cleavage (Fig. 3A). The addition of 2 eq of Cu²⁺ (40 μM), but no ascorbic acid, to the cleavage reaction produced distinct differences in the relative amounts of α₁-, α₂-, and β-cleavage products (Fig. 3C). We report only peak integration of C-terminal fragments because co-elution of N-terminal cleavage fragments prevented reliable quantification of the...
on the same peptide, the observed levels of N- and C-terminal fragments do not necessarily match, notably between N2 and C2 fragments.

To evaluate the rate at which ADAM8 cleaves MoPrP, we performed a time trial over the course of 12 h (Fig. 4C). Primary cleavage was nearly complete by 4 h; however, the level of C2 continued to drop while the level of α2-C1 increased, likely due to C2 peptide fragments being subsequently cleaved again at the α1-site.

Zn2+ Modulates ADAM8 Cleavage of MoPrP in a Pattern Different from That of Cu2+—MoPrP is capable of coordinating Zn2+ in the octarepeat domain, albeit with a weaker affinity than that for Cu2+. However, extracellular levels of Zn2+ are significantly higher compared with Cu2+, suggesting that PrPC may nevertheless coordinate Zn2+ in vivo (30). Additionally, PrPC is involved in zinc uptake into neurons (31). To evaluate whether Zn2+ influences ADAM8 cleavage of MoPrP, we performed an ADAM8 cleavage assay with 20 μM MoPrP and 80 μM Zn2+ (Fig. 5). MoPrP can coordinate only one Zn2+ ion; the added 4 eq are the maximum possible without lowering MoPrP solubility. The addition of Zn2+ to the cleavage reaction resulted in a similar reduction of C2 to that seen with Cu2+; however, the relative proportions of α1- and α2-C1 were reversed. This suggests that zinc suppresses β-cleavage in the octarepeat domain, where the metal ion is known to bind, but does not influence the proportions of α-cleavage relative to the case with enzyme alone.

Cu2+ Modulates ADAM8 α1-Cleavage of MoPrP through His-110 and α2-Cleavage through the Octarepeat Domain—The data above suggest that copper coordination to His-110 suppresses α1-cleavage. To test this hypothesis, we mutated MoPrP His-110 to tyrosine, thereby eliminating Cu2+ coordination at that residue. Surprisingly, as opposed to removing the copper blockage to α1-cleavage, the H110Y mutation actually reduced α1-cleavage (Fig. 6A), which suggests that His is part of the consensus sequence for ADAM8 activity. As with the wild type, the addition of copper reduced β-cleavage and enhanced general α-cleavage, with the latter primarily as α2-products (Fig. 6A).

We next assayed ADAM8 cleavage of MoPrP(90–230), which lacks the N terminus, including the octarepeat domain (Fig. 6B). Analysis of the relatively low molecular weight N-terminal cleavage fragments is provided for MoPrP(90–230) experiments due to their superior HPLC separation compared with C-terminal fragments. Although cleavage fragments corresponding to both α1- and α2-cleavage were observed in the absence of Cu2+, generally less cleavage was seen as reflected in the amount of unreacted full-length protein. The addition of 2 eq of Cu2+ resulted in a decrease in α1-cleavage, similar to wild-type MoPrP, whereas α2-cleavage levels were unaffected by the addition Cu2+. Together, these data suggest that α2-cleavage is enhanced by copper occupation of the octarepeat domain in the wild-type protein, perhaps through a non-local interaction (see “Discussion”).

Pathogenic Mutants MoPrP E199K and D177N Exhibit Decreased ADAM8 α1-Cleavage upon Addition of Cu2+ Relative to Wild-type MoPrP—Recent work suggests that Zn2+ and Cu2+ promote a tertiary interaction between the metal-occupied

N-terminal peaks. Interestingly, the addition of Cu2+ to the cleavage reaction appeared to reduce the amounts of α1- and β-cleavage while increasing the amount of α2-cleavage. The percent of reacted MoPrP remained relatively unchanged at >85% with or without the addition of Cu2+ (Fig. 3B). As a control, the entire LC traces in Fig. 3A were integrated to ensure that the total amount of protein observed for each cleavage reaction was approximately the same.

To further understand the role that Cu2+ plays in ADAM8 cleavage, we performed a titration from 0 to 8 eq of Cu2+ (Fig. 4, A and B, N- and C-terminal fragments, respectively). In the absence of Cu2+, cleavage was dominated by α1- and β-cleavage, as noted above. However, the addition of 1 eq of Cu2+, which binds primarily to the octarepeat domain, reduced the amount of C2 protein observed by a factor of 2 while increasing the amounts of both α1- and α2-C1 fragments. Additional equivalents of Cu2+ resulted in increasing amounts of α2-C1 while reducing the level of α1-C1. This suggests that copper coordination at His-110 suppresses α1-cleavage. We note that because both α- and β-cleavage events can occur
N-terminal octarepeats and the folded C-terminal domain (24, 32). In addition, two C-terminal pathogenic mutants in familial prion disease, MoPrP E199K and D177N, destabilize this interdomain interaction. Because Cu²⁺ modulates α₂-cleavage (see above), we wanted to evaluate the consequence of the E199K and D177N mutations on ADAM8 cleavage with and without added copper. Analysis of ADAM8 cleavage by LC/MS revealed that in the apo-state, MoPrP E199K and D177N behaved like wild-type MoPrP (Fig. 7A), with both mutants giving a preponderance of C₂ product. When Cu²⁺ was added to both wild-type MoPrP and the mutant species, C₂ was suppressed relative to C₁; however, the ratios of C₁- and C₂-products depended on the specific protein. As shown in Fig. 7B, in the absence of copper (apo-state), the C₂/C₁ product ratio was constant. However, with 2 eq of copper, the mutant species showed systematically less C₂ relative to C₁-

ADAM10 and ADAM17 Cleave MoPrP at a Novel Location, Termed C₃-Cleavage—In addition to ADAM8, both ADAM10 and ADAM17 are implicated in C₃-cleavage of MoPrP (8). To investigate the role these enzymes play in MoPrP cleavage, we performed the same LC/MS assay we used for ADAM8 with recombinant ADAM10 and ADAM17 (Fig. 8A). Analysis for both N- and C-terminal fragments is reported due to good HPLC separation of all fragments. We buffered the cleavage reaction at both physiological pH 7.4 and enzymatically optimal pH 9. After a 6-h reaction, neither ADAM10 nor ADAM17 cleaved MoPrP at the purported site of C₂-cleavage, Lys-109→His-110. Instead, the proteases cleaved MoPrP several amino acids more C-terminal at Ala-119→Val-120, which will be referred to as C₃-cleavage. Additionally, ADAM10 cleaved MoPrP at Gly-227→Arg-228, which has been previously reported as sheddase activity, releasing PrP from its membrane anchor (9). We refer to the released PrP product as N₃.
Cu²⁺ nor Zn²⁺ affected ADAM10 or ADAM17 cleavage of MoPrP (data not shown).

Given the success of MoPrP H110Y at blocking ADAM8-mediated α₁-cleavage, we sought to create an additional MoPrP mutant that could block ADAM10 and ADAM17 α₃-cleavage. Previous work with peptide libraries has mapped out the contribution of each amino acid in the active site to enhancing or reducing cleavage by ADAM10 and ADAM17 (33). Aspartic acid at any location in the P₅ to P₄ position of the cleavage site showed greatly reduced ADAM10 and ADAM17 cleavage; we therefore mutated MoPrP Val-120 to aspartic acid in an attempt to block cleavage at Ala-119→Val-120. After a 24-h reaction, MoPrP V120D gave an 80–90% reduction in α₃-cleavage from either ADAM10 or ADAM17 (Fig. 8B). ADAM10 was still able to cleave MoPrP V120D at Gly-227→Arg-228, producing N3 at levels comparable to wild-type MoPrP. Using the same strategy, we mutated Arg-228 to aspartic acid to block ADAM10 cleavage at Gly-227→Asp-228 and the consequent formation of N3. After a 24-h incubation with ADAM10, we saw no cleavage at Gly-227→Asp-228, whereas ADAM10 cleavage at Ala-119→Val-120 remained unaffected. These findings show that the strategy of X→Asp mutation successfully blocks selective ADAM10/ADAM17 activity.

Embryonic Lethal MoPrP Mutant ΔCR Lacking Residues 105–125 Produces Alternative Cleavage Patterns When Exposed to ADAM8, ADAM10, and ADAM17—Although MoPrP knock-out mice are viable, mice expressing a MoPrP mutant with residues 105–125 deleted (MoPrPΔCR) do not reach maturity (Ref. 34; see also the work of Baumann et al. (35), in which residues 94–134 were deleted). The reasons for the lethal phenotype are not known, although it has been proposed that amino acids 105–125 of MoPrP are critical for an interaction with a potential PrP binding partner (34). Given our findings that ADAM8, ADAM10, and ADAM17 all cleave MoPrP within this region, we hypothesized that MoPrP ΔCR would alter all forms of N-cleavage. We therefore determined the cleavage patterns for MoPrPΔCR mediated by all three proteases (Fig. 9). Wild-type MoPrP cleaved by ADAM8 underwent N₁- and N₂-cleavage and β-cleavage;
however, MoPrP ΔCR, lacking the α₁- and α₃-cleavage sites, showed only β-cleavage as reflected by the N2 product. ADAM10 maintained cleavage of MoPrP ΔCR near the C terminus (N3 product); however, no fragments corresponding to α₅-cleavage were observed. Instead, a small amount of peptide resulting from cleavage at Tyr-127 ↓ Met-128 was produced (wild-type MoPrP sequence). Likewise, ADAM17 gave an alternative cleavage location at Gly-130 ↓ Ser-131. The new cleavage locations from ADAM10 and ADAM17 are both quite close to the deleted region, but the efficiency is low relative to the wild type.

**DISCUSSION**

α-Cleavage and β-cleavage of Prp⁴C are important proteolytic processing events that release bioactive protein fragments. With few exceptions (7, 9, 36), the identification of Prp⁴C cleavage products has been determined by Western blotting, which lacks the power to resolve fragment masses at atomic mass unit resolution. Subtle differences in fragment masses may not be distinguishable, leading to low resolution and perhaps an oversimplified understanding of Prp⁴C processing. Here, we applied LC/MS to evaluate the detailed reactions of copper/ascorbate, ADAM8, ADAM10, and ADAM17 on full-length Prp⁴C to systematically analyze MoPrP cleavage fragments.

Our primary findings are encapsulated with the following observations: 1) Combined ascorbic acid and Cu²⁺ generate ROS that cleave MoPrP within the octarepeat domain (α₁-cleavage). ADAM8 still cleaved ΔCR within the octarepeat domain (N2), but no α₁- or α₂-cleavage was observed. ADAM10 cleaved ΔCR at Gly-227 ↓ Arg-228 (wild-type amino acid indexing), producing the N3 fragment, but cleavage at Ala-119 ↓ Val-120 was abolished. Instead, a small amount of peptide fragment corresponding to cleavage at Tyr-127 ↓ Met-128 was observed. ADAM17 also did not cleave ΔCR at Ala-119 ↓ Val-120 but instead at Gly-130 ↓ Ser-131. 2) ADAM8 cleaves MoPrP at H110Y, the previously identified location of α₁-cleavage in brain extract. Additional cleavage is also observed around Ala-116 (α₂-cleavage) and within the octarepeat domain (β-cleavage) in a Cu²⁺- and Zn²⁺-dependent fashion. 3) ADAM10 and ADAM17 cleave MoPrP at Val-120, and ADAM10 also cleaves MoPrP at Arg-228, which releases the protein from its membrane anchor. 4) The toxic mutants MoPrP E199K and D177N (mouse numbering) and
MoPrP ΔCR all produce altered ADAM cleavage patterns. Notably, the cleavage patterns observed for E199K and D177N are influenced by the presence of Cu\(^{2+}\). 5) The H110Y, V120D, and R228D mutants block ADAM cleavage at their respective locations.

Our results are summarized in Fig. 10. Experiments involving ADAM8 are detailed on the left. The thickness of the arrows corresponds to the relative levels of cleavage yield at the indicated locations in the absence and presence of Cu\(^{2+}\) and Zn\(^{2+}\). The overall cleavage locations for ADAM8, ADAM10, and ADAM17 are depicted on the right. Additionally, dashed lines denote those cleavage locations blocked by our engineered MoPrP mutants.

Current thinking holds that PrPC undergoes \(\alpha\)- and \(\beta\)-cleavage by distinct mechanisms. \(\alpha\)-Cleavage is a normal processing event observed in brain extract that occurs between Lys-109 and His-110, likely resulting from the action of ADAM8, ADAM10, or ADAM17. However, \(\beta\)-cleavage is an aberrant reaction, seen at abnormally high levels in Creutzfeldt-Jakob disease brain extract, and results from the copper-mediated production of ROS that chemically cleave the polypeptide backbone at the end of the octarepeat domain. Our findings suggest a revision of this elementary scheme. First, we found that \(\beta\)-cleavage may also be enzymatic, with ADAM8 acting with equivalent efficiency at the \(\alpha\)- and \(\beta\)-sites. Moreover, ADAM8-driven \(\beta\)-cleavage does not correspond to a single site but instead takes place between each Pro-His bond in the octarepeat domain. Second, in the absence of a reducing agent, we found that copper actually suppresses \(\beta\)-cleavage. We propose that by binding with high affinity to N-terminal His residues within the octarepeat and at His-110, Cu\(^{2+}\) prevents the polypeptide substrate from entering the enzyme active site, thereby suppressing \(\beta\)- and \(\alpha\)-cleavage, respectively. Zn\(^{2+}\) binds only to the octarepeat domain and accordingly suppresses only \(\beta\)-cleavage. Finally, we found that \(\alpha\)-cleavage does not take place at a single site but instead occurs at one of three nearby sites depending on the specific enzyme. ADAM8 produces \(\alpha_1\)- and \(\alpha_2\)-cleavage, preceding His-110 and Ala-117 (palindromic region), respectively, whereas ADAM10 and ADAM17 produce \(\alpha_3\)-cleavage at Val-120. \(\alpha\)-Cleavage occurring at multiple sites provides an explanation for why the entire palindromic region of PrPC had to be deleted to fully block cleavage in vivo (12). Deletions of shorter regions that encompass only \(\alpha_1\), \(\alpha_2\), or \(\alpha_3\) only partially suppressed cleavage.

Spevacek et al. (24) discovered a novel N-terminal/C-terminal interaction in PrPC promoted by zinc. Specifically, Zn\(^{2+}\) binds to the octarepeat domain, which then docks against a conserved negatively charged C-terminal surface formed by helices 2 and 3. Pathogenic mutations localized to these helices tend to reduce the strength of this tertiary interaction. The
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proteolysis results reported here suggest that the zinc-induced fold increases the sensitivity to \( \alpha_3 \)-cleavage (Fig. 5). As shown in Fig. 6A, the addition of 2 eq of Cu\( ^{2+} \) to wild-type PrP\( ^{C} \) (with 1 eq taken up in the octarepeat domain) with His coordination like that of Zn\( ^{2+} \) promotes \( \alpha_2 \)-cleavage. However, this effect is muted in PrP(90–230) (Fig. 6B), which lacks the octarepeat domain. Moreover, PrP\( ^{C} \) with pathogenic mutations (Fig. 7B) exhibits a decrease in \( \alpha_2 \)-cleavage relative to \( \alpha_2 \)-cleavage but only in the presence of Cu\( ^{2+} \). These data suggest that the tertiary structure promoted by metal ion coordination exposes the palindromic region of PrP\( ^{C} \), which resides between the octarepeats and the folded C-terminal domain, thereby enhancing \( \alpha_2 \)-cleavage.

It is interesting to consider PrP\( ^{C} \) as a proprotein, with \( \alpha \)-cleavage required to release the active N1 peptide. Evolving literature from the Checler laboratory (13) shows that N1 is protective and acts by suppressing apoptosis, possibly through inhibition of caspase-3 or other pro-apoptotic agents. The mechanism by which extracellular N1 affects these intracellular processes is unknown but apparently does not require N1 internalization. The N1 peptide may also play an important role in slowing the progression of Alzheimer disease. Harris and colleagues (37) demonstrated that N1 binds with high affinity to A\( \beta \) oligomers, thereby reducing A\( \beta \) toxicity in neuron culture and animal assays. A\( \beta \) oligomer binding takes place at two locations in N1: residues 23–31 and 95–105. All \( \alpha \)-cleavage points identified by our studies are C-terminal of segment 95–105 and likely preserve A\( \beta \) affinity among the three N1 variants. However, this has not been directly tested. Copper was previously linked to \( \beta \)-cleavage, which produces N2 lacking residues 95–105 and thus might be considered as a species that enhances the events leading to \( \beta \)-driven neurodegeneration. However, our findings here suggest a different scenario. As summarized in Fig. 10, both copper (under nonreducing conditions) and zinc suppress \( \beta \)-cleavage, and copper enhances \( \alpha_2 \)-cleavage. Consequently, these metal ions may be important regulators in the production of N1. If so, our findings may help explain the often conflicting results of copper chelation therapies in the treatment of Alzheimer disease (38–41).

PrP\( ^{C} \) is found in various biologically active forms: full-length protein anchored to the extracellular membrane, released full-length protein shed from the membrane, \( \alpha \)-cleaved protein (N1 and C1 fragments), and \( \beta \)-cleaved protein (N2 and C2 fragments). At the present time, there is little understanding with regard to how alteration in cleavage patterns plays into general PrP\( ^{C} \) function and prion disease. For example, as noted above, the lethality of MoPrP ACR is attributed to a loss of molecular recognition of an unknown PrP\( ^{C} \) binding partner (34) or through promotion of spontaneous ion channeling (42). However, it is also possible that deletion of the CR region, which spans the \( \alpha_3 \)-, \( \alpha_2 \)-, and \( \alpha_3 \)-cleavage sites, exerts its lethal phenotype by fully blocking native \( \alpha \)-cleavage. In this scenario, released N1 may be essential for myelination and other components of neurological development. Similarly, proteolytic shedding activity (as distinct from GPI anchor cleavage) may be important for maintaining levels of free PrP\( ^{C} \), with potential roles in neuron development and maintenance (10, 23). In our work here, beyond identification of specific enzyme cleavage sites, we also developed and tested \( X \rightarrow \text{Tyr or Asp point mutations that selectively inhibit ADAM8, ADAM10, and ADAM17 proteolysis, thus suppressing }\alpha_3\text{-cleavage, sheddase, and }\alpha_2\text{-cleavage, respectively. In cellular and transgenic animal studies, these mutants could serve as important new tools for assessing the relative roles of PrP\( ^{C} \) cleavage products.}

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