

Reactive Oxygen Species-mediated β -Cleavage of the Prion Protein in the Cellular Response to Oxidative Stress*

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The cellular prion protein (PrP^C) is critical for the development of prion diseases. However, the physiological role of PrP^C is less clear, although a role in the cellular resistance to oxidative stress has been proposed. PrP^C is cleaved at the end of the copper-binding octapeptide repeats through the action of reactive oxygen species (ROS), a process termed β -cleavage. Here we show that ROS-mediated β -cleavage of cell surface PrP^C occurs within minutes and was inhibited by the hydroxyl radical quencher dimethyl sulfoxide and by an antibody against the octapeptide repeats. A construct of PrP lacking the octapeptide repeats, PrP Δ oct, failed to undergo ROS-mediated β -cleavage, as did two mutant forms of PrP, PG14 and A116V, associated with human prion diseases. As compared with cells expressing wild type PrP, when challenged with H₂O₂ and Cu²⁺, cells expressing PrP Δ oct, PG14, or A116V had reduced viability and glutathione peroxidase activity and increased intracellular free radicals. Thus, lack of ROS-mediated β -cleavage of PrP correlated with the sensitivity of the cells to oxidative stress. These data indicate that the β -cleavage of PrP^C is an early and critical event in the mechanism by which PrP protects cells against oxidative stress.

Prion diseases or transmissible spongiform encephalopathies are a group of neurodegenerative disorders including scrapie in sheep, bovine spongiform encephalopathy in cattle, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler-Scheinker disease in humans (1). In prion diseases, the normal cellular form of the prion protein (PrP^C)⁴ undergoes a conformational conversion to the β -sheet-rich scrapie isoform (PrP^{Sc}) that is partially resistant to protease digestion. Although PrP^C is critical for the development of prion disease through its conversion into PrP^{Sc} (2, 3), the physiological role of PrP^C is less clear, and thus it is uncertain whether prion diseases are, in part, due to the loss of a normal neuroprotective function of PrP^C (4). In the brains of animals at the terminal stage of illness, there is a marked decrease of PrP^C, supporting the hypothesis that loss of function of PrP^C may play a role in the pathogenesis of prion diseases (5).

Among the neuroprotective functions of PrP^C are roles in copper homeostasis and the cellular resistance to oxidative stress (6, 7). PrP^C

binds Cu²⁺ ions, primarily within the N-terminal octapeptide repeats (8–10), undergoes endocytosis upon exposure of cells to Cu²⁺ (11, 12), and modulates neuronal Cu²⁺ content (13), implicating PrP^C in cellular copper metabolism. Cells deficient in PrP^C are less viable in culture compared with cells expressing wild-type PrP (wtPrP) and are more susceptible to oxidative damage and toxicity caused by reactive oxygen species (ROS) (14–17), implicating PrP^C in the cellular response to oxidative stress. However, the mechanism by which PrP^C mediates this protective effect is not known.

PrP^C is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that undergoes a variety of proteolytic processing events. The protein can be cleaved at amino acids 110 and 111 to produce a 17-kDa C-terminal fragment C1 and a corresponding N-terminal fragment N1 (18–21). This processing has been referred to as α -cleavage (22) and may be mediated by ADAM 10 and ADAM 17, members of the ADAM (a disintegrin and metalloprotease) family (23). PrP^C can also be cleaved within or adjacent to the octapeptide repeats to generate a 21-kDa C-terminal fragment C2 and the corresponding N-terminal fragment N2 (18, 21, 24). This cleavage event appears to be mediated by ROS (25) and has been termed β -cleavage (22). In addition, we have recently shown that PrP^C is proteolytically shed from the cell surface by a zinc metalloprotease that has similar properties to the α -secretase cleavage of the Alzheimer amyloid precursor protein (26). Understanding the role of these proteolytic cleavages and of the fragments generated is critical to a full understanding of the biological functions of PrP and may also impact on the role of the protein in prion diseases.

However, the role of the β -cleavage in the function of PrP^C has not been addressed. In the current study, we show that PrP expressed in the human neuroblastoma SH-SY5Y cell line undergoes both α - and β -cleavage and that β -cleavage is increased upon exposure of the cells to ROS, occurs at the cell surface, and can be inhibited by a free radical quencher. We also show that β -cleavage does not occur in a mutant of PrP that lacks the octapeptide repeats (PrP Δ oct) nor in two disease-associated mutants of PrP (PG14 and A116V). This lack of β -cleavage of the PrP mutants correlates with the sensitivity of cells to ROS, indicating that this cleavage event may be part of the mechanism by which PrP^C protects cells against oxidative stress.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Cell Culture—The construction of wtPrP, PrP Δ oct, and PG14 in pIRESneo has been described previously (12). A116V was generated from wtPrP using the QuikChange site-directed mutagenesis kit (Stratagene) with the following primers: sense, 5'-CA-GGGGCTGCGGTAGCTGGGGGCAGTAG-3'; antisense, 5'-CTAC-TGCCCCAGCTACCGCAGCCCCCTG-3'. The resulting construct was verified by DNA sequencing. Human neuroblastoma SH-SY5Y cells were cultured and transfected by electroporation, and pooled, stable cell lines were obtained by antibiotic selection as described previously (27). Copper was routinely administered to the cells as CuSO₄ in the presence

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⁴ The abbreviations used are: PrP^C, cellular form of the prion protein; PrP^{Sc}, infectious, protease resistant form of PrP; ALLM, N-acetyl-Leu-Leu-Met-aldehyde; FCS, fetal calf serum; PBS, phosphate-buffered saline; PNGase F, Peptide:N-glycosidase F; ROS, reactive oxygen species; wtPrP, wild type PrP; GPI, glycosylphosphatidylinositol.

of fetal calf serum (FCS) to provide a source of both albumin and histidine for the Cu^{2+} to complex to. When the cells had reached confluence, the monolayer was washed twice with Opti-MEM before incubation with the relevant compounds for the specified periods of exposure in Opti-MEM. Cells were harvested into phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4 , 2.7 mM Na_2HPO_4 , 150 mM NaCl, pH 7.4) pelleted by centrifugation at $1000 \times g$ for 5 min, and resuspended in lysis buffer (10 mM Tris/HCl, pH 7.8, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 10 mM EDTA, supplemented with complete protease inhibitor mixture). The protein content of each lysate was determined using bicinchoninic acid in a microtiter plate assay with bovine serum albumin as a standard (28).

Surface Biotinylation and Immunoprecipitation—Cells at confluence were incubated for 1 h at 4°C with 0.5 mg/ml Biotin sulfo-*N*-hydroxysuccinimide (NHS), washed three times with 50 mM glycine to quench the biotinylation reaction, and then incubated for various times at 37°C in the absence or presence of $10\ \mu\text{M}$ CuSO_4 and $100\ \mu\text{M}$ H_2O_2 in Opti-MEM. Cell lysates were made 1% (w/v) with respect to *N*-lauroylsarcosine and incubated for 30 min with 0.5% (w/v) protein A-Sepharose. The protein A-Sepharose was pelleted by centrifugation for 1 min at $13,000 \times g$, and the supernatant was incubated overnight at 4°C with 0.1% (v/v) 3F4 antibody. Protein A-Sepharose was added to 0.5% (w/v), and incubation continued for 1 h. The immunocomplexes were pelleted by centrifugation at $13,000 \times g$ for 1 min and washed three times with 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris/HCl, pH 8.0, 1% (v/v) Nonidet P-40. The remaining pellet was resuspended in dissociation buffer for analysis by SDS-PAGE and Western blot. To measure copper-induced endocytosis, biotin-labeled cells were incubated for 30 min at 37°C in the absence or presence of $100\ \mu\text{M}$ CuSO_4 presented as a histidine chelate. PrP remaining at the cell surface was removed by digestion with trypsin as previously described (12).

Enzymic Deglycosylation, SDS-PAGE, and Western Blot Analysis—Samples were deglycosylated with peptide-*N*-glycosidase F (PNGase F) (Europa Bioproducts, Ely, UK) for 16 h at 37°C as described previously (27). Where indicated, samples were digested with 5 $\mu\text{g}/\text{ml}$ proteinase K for 1 h at 37°C . Samples (containing 15 μg of total protein) or immunoprecipitates were resolved by electrophoresis through 14.5% polyacrylamide gels. For Western blot analysis, resolved proteins were transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked by incubation for 1 h with PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) dried milk powder. Incubations with primary antibodies 3F4 (Signet Laboratories, Inc., Dedham, MA), SAF32 (Cayman Chemical, Ann Arbor, MI), 6H4 (Prionics, Zurich, Switzerland), or anti-actin and peroxidase-conjugated secondary antibodies were performed for 1 h in the same buffer. Incubation with peroxidase-conjugated streptavidin was performed for 1 h in PBS containing 0.1% (v/v) Tween 20. Bound peroxidase conjugates were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences).

Recombinant Calpain Activity Assay—The activity of 20 nM recombinant Calpain-2 (Calbiochem) was measured using 5 μM (5-aminomethylfluorescein)-Gly-Gly-Gly-Gln-Leu-Tyr-Gly-Gly(*N* ^{β} -(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid)-Arg-Arg-Lys(tetramethylrhodamine) NH_2 (a gift from GlaxoSmithKline, Harlow, UK) in 60 mM imidazole/HCl, 5 mM L-cysteine, 2.5 mM glutathione (reduced), and 5 mM CaCl_2 , pH 7.3. The broad spectrum calpain inhibitor *N*-acetyl-Leu-Leu-Met-aldehyde (ALLM) resuspended in either Me_2SO or EtOH was added at 1, 10, or 100 μM . Activity was recorded as fluorescence released following cleavage of the substrate over 1 h using a Synergy HT (Bio-Tek) with excitation at 485 nm and emission at 528 nm. The data were expressed as the percentage inhibition of activity compared with the uninhibited control.

Immunofluorescence Microscopy—Cells were seeded onto coverslips and grown to 50% confluence. The fate of cell surface PrP^C was monitored by prelabeling cells with antibody 3F4 for 30 min at 4°C . Cells were then incubated in Dulbecco's PBS in the presence or absence of 10 $\mu\text{g}/\text{ml}$ *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C for 30 min at 37°C . Cells were then fixed with 4% (v/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde in PBS for 15 min and blocked overnight in PBS containing 3% (v/v) goat serum. Finally, coverslips were incubated with AlexaFluor 488[®] rabbit anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) for 1 h and mounted on slides using fluoromount G mounting medium (SouthernBiotech). Individual cells were visualized using a DeltaVision Optical Restoration Microscopy System (Applied Precision Inc.). Data were collected from 30–40 0.1- μm -thick optical sections, and three-dimensional data sets were deconvolved using the softWoRx program (Applied Precision Inc.). The images represent individual Z-slices corresponding to the middle of the cell.

Assessment of Cell Number by Hoescht 33342 Staining—Cells ($1 \times 10^4/\text{well}$) in 96-well tissue culture plates were cultured overnight in serum-free medium. After 24 h, this was replaced with 5% FCS-containing medium supplemented with H_2O_2 (100 μM), CuSO_4 (8 μM), or both reagents. After a further 48 h, the cells were fixed in 70% ethanol at room temperature for 5 min, and the adherent cell monolayers were stained with the DNA-binding fluorochrome Hoescht 33342 (8.8 μM). Once dry, the fluorescence of each well was measured on a Synergy HT (Bio-Tek) (350-nm excitation and 450-nm emission wavelengths) in order to determine the cell number in each well.

Measurement of Intracellular Oxidative Activity and Glutathione Peroxidase Activity—The level of intracellular free radicals was determined following exposure of the cells to H_2O_2 (100 μM), CuSO_4 (8 μM), or both reagents in 5% FCS-containing medium for 6 h using 100 μM dihydrodichlorofluorescein diacetate as described previously (17). Glutathione peroxidase activity was measured using 50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0, 1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH, 1 mM glutathione, and 1 unit/ml glutathione reductase at room temperature upon the addition of 0.1 ml of cumene hydroperoxide (1.5 mM) as described previously (17).

Statistical Analysis—All analyses were subject to Kruskal-Wallis nonparametric one-way analysis of variance. $p < 0.001$ were considered highly significant. Changes in cell number, intracellular radical generation, and glutathione peroxidase activity in the cells expressing the mutant PrPs are all compared against wtPrP-expressing cells.

RESULTS

PrP^C in SH-SY5Y Cells Is Subject to α - and β -Cleavages—The proteolytic processing of murine PrP^C containing the 3F4 epitope (wtPrP) stably expressed in the human neuroblastoma SH-SY5Y cell line was examined using antibodies that recognize different epitopes in the protein (Fig. 1). To remove the problems of interpreting the immunoblots because of the variable glycosylation states of full-length PrP and of the C-terminal fragments, samples were deglycosylated prior to immunoblotting. Antibody SAF32, which recognizes an epitope within the octapeptide repeats, as expected detected full-length PrP but neither the C1 nor C2 fragments in the cell lysate (Fig. 1B). Antibody 3F4, which recognizes the engineered epitope MHKM (residues 108–111 of murine PrP), detected both full-length PrP and the C2 fragment of molecular mass 21 kDa but not the C1 fragment, since α -cleavage destroys the epitope recognized by this antibody (Fig. 1C) (20, 21). Antibody 6H4, which recognizes an epitope in the C-terminal half of the protein (residues 144–152), detected full-length PrP, C2, and the C1

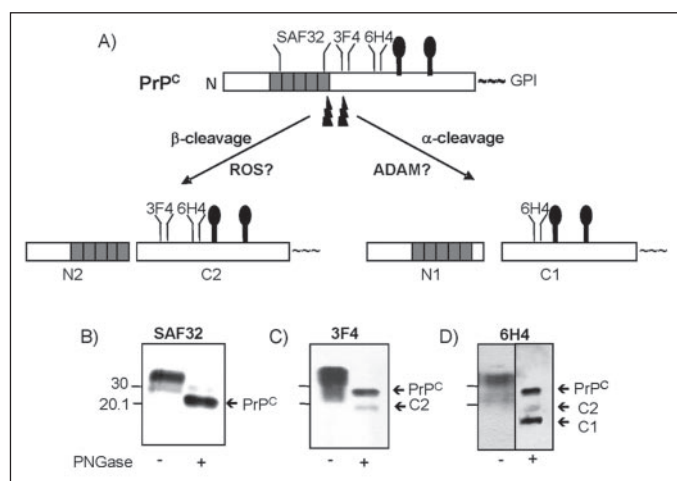


FIGURE 1. PrP^C in SH-SY5Y cells is subject to both α - and β -cleavages. A, schematic diagram of the proteolysis of PrP^C and the epitopes recognized by the antibodies used in this study. Mature, full-length PrP^C is shown with its C-terminal GPI anchor, two N-linked glycosylation sites (residues 180 and 196, lollipops), and the octapeptide repeat region (shaded). The epitopes for antibody SAF32 (within the octapeptide repeats), 3F4 (residues 108–111), and 6H4 (residues 144–152) are indicated. The two cleavage sites generating N1/C1 (α -cleavage) and N2/C2 (β -cleavage) are shown by jagged arrows. C2 is recognized by both 3F4 and 6H4, whereas C1 is only recognized by 6H4. B–D, lysates (15 μ g) from SH-SY5Y cells expressing wtPrP were incubated in the absence or presence of PNGase F and immunoblotted with SAF32 (B), 3F4 (C), and 6H4 (25 μ g of lysate) (D). Molecular mass markers (kDa) are indicated on the left.

fragment of molecular mass 17 kDa (Fig. 1D). Although the C1 fragment was detected at a similar level of intensity as full-length PrP, the C2 fragment was present at a significantly lower level. These data indicate that in SH-SY5Y cells PrP^C is subject to both α - and β -proteolytic cleavages to generate C1 and C2 fragments, respectively, as reported for other cell lines and in brain tissue (19–22).

β -Cleavage of PrP^C Is Up-regulated When Cells Are Subjected to Oxidative Stress—Since there is some evidence to suggest that β -cleavage of PrP^C is mediated by ROS (25), we examined the effect of H₂O₂ and Cu²⁺ on the formation of the C2 fragment (Fig. 2). Changing the cell medium from serum-containing to serum-free Opti-MEM caused an increase in the production of C2 (Fig. 2, A and D) due to the removal of survival factors on withdrawal of the serum from the medium (15). In the presence of 100 μ M H₂O₂ and 10 μ M Cu²⁺, there was a further increase in the level of C2 above that observed in the serum-free medium-treated cells within 10 min (Fig. 2, A and D). In contrast to the changes in C2, the levels of neither full-length PrP (Fig. 2, B and E) nor the C1 fragment (Fig. 2, B and F) altered with the removal of serum or upon treatment of the cells with H₂O₂ and Cu²⁺. These observations indicate that ROS increased the production of C2 via β -cleavage but had no effect on α -cleavage. To confirm that β -cleavage of PrP^C is indeed a ROS-mediated event, the effect of the hydroxyl radical quencher Me₂SO was examined (Fig. 3). When cells expressing wtPrP were incubated in serum-free Opti-MEM in the presence of Me₂SO, there was a dose-dependent reduction in the production of C2 (Fig. 3, A and C), consistent with β -cleavage being a ROS-mediated process.

ROS-mediated β -Cleavage of PrP^C Occurs at the Cell Surface—To determine whether cell surface PrP^C was subject to ROS-mediated β -cleavage, cells expressing wtPrP were first surface-biotinylated prior to treatment with 100 μ M H₂O₂ and 10 μ M Cu²⁺ (Fig. 4). Following immunoprecipitation of PrP with antibody 3F4, biotinylated full-length PrP and C2 were visualized by immunoblotting with peroxidase-conjugated streptavidin. Immediately following biotinylation, negligible biotinylated C2 was detected in the cell lysate, although significant amounts of biotinylated full-length PrP were present. However, an

increase in the level of biotinylated C2 fragment was clearly evident following incubation of the cells in serum-free Opti-MEM for 10 min, and this was further increased upon treatment of the cells with H₂O₂ and Cu²⁺, indicating that C2 is formed from PrP^C exposed at the cell surface.

The Octapeptide Repeats Are Required for the ROS-mediated β -Cleavage of PrP^C—To determine whether ROS-mediated β -cleavage of PrP^C required the octapeptide repeats, we examined the proteolytic processing of PrP Δ oct that lacks the copper-binding octapeptide repeat region (12) (Fig. 5A). Lysates from cells expressing PrP Δ oct were subjected to immunoblot analysis with antibodies SAF32, 3F4, and 6H4 (Fig. 5, B–D). SAF32 failed to detect PrP Δ oct, since this mutant lacks the epitope for this antibody but was detected by 3F4 and 6H4. Although antibody 6H4 clearly detected the C1 fragment in cells expressing PrP Δ oct, neither antibody 6H4 nor 3F4 detected the C2 fragment. These data indicate that the octapeptide repeats are required for PrP^C to undergo β -cleavage.

ROS-mediated β -Cleavage Is Defective in Two Disease-associated Mutants of PrP—We examined next the proteolytic processing of two disease-associated mutants of PrP. PG14 contains an extra nine copies of the octapeptide repeat and is associated with familial human prion disease (29, 30) and A116V, in which Ala¹¹⁶ (murine PrP numbering, equivalent to Ala¹¹⁷ in human PrP) is mutated to Val and is associated with Gerstmann-Sträussler-Scheinker disease (31) (Fig. 5A). Lysates from cells expressing the two mutants were subjected to immunoblot analysis with antibodies SAF32, 3F4, and 6H4 (Fig. 5, B–D). Although in cells expressing either PG14 or A116V, all three antibodies detected full-length protein, and 6H4 detected the C1 fragment, there was no detection of the C2 fragment in either cell line by antibody 3F4 or 6H4 even after prolonged exposure of the immunoblots (Fig. 5, B and D). Even upon treatment of the cells expressing PG14 or A116V with H₂O₂ and Cu²⁺ for up to 60 min, there was no evidence for the production of C2, whereas under identical conditions, C2 was clearly formed in cells expressing wtPrP (data not shown). These data indicate that in cells expressing two disease-associated mutants of PrP, although C1 is formed normally, C2 is not formed upon exposure of the cells to ROS.

One possible explanation for the lack of ROS-mediated β -cleavage in cells expressing either PG14 or A116V is that the mutants fail to traffic to the cell surface where this processing occurs. Previously, however, we (12) and others (32) have shown by surface biotinylation and immunofluorescence microscopy that PG14 is localized at the cell surface. Although the A116V mutant is expressed at a lower level than wtPrP in the SH-SY5Y cells (Fig. 6A), the amount of this mutant at the cell surface as revealed by surface biotinylation was very similar to that of wtPrP (Fig. 6B). The cell surface localization of A116V was confirmed by immunofluorescence microscopy (Fig. 6C). Like wtPrP, the A116V construct gave a similar pattern of cell surface staining, which could be abolished by incubation of the cells with bacterial phosphatidylinositol-specific phospholipase C, which cleaves the GPI anchor, releasing the protein from the membrane. Since neither the PrP Δ oct nor the PG14 mutants are endocytosed when cells are exposed to Cu²⁺ ions (12), we considered that the lack of β -cleavage may correlate with a deficiency in copper-mediated endocytosis. However, when cells expressing A116V were exposed to a concentration of Cu²⁺ ions sufficient to promote endocytosis of wtPrP (12), this mutant was rapidly endocytosed (Fig. 6D). Thus, the inability of the A116V and PG14 mutants to reach the cell surface does not account for the lack of ROS-mediated β -cleavage of these mutants, and failure to undergo β -cleavage does not correlate with a deficiency in copper-mediated endocytosis.

FIGURE 2. Copper and H₂O₂ up-regulate the formation of C2. SH-SY5Y cells stably expressing wtPrP were either maintained in 10% FCS-containing Dulbecco's modified Eagle's medium (Serum), maintained in Opti-MEM medium, or treated with 10 μ M CuSO₄ and 100 μ M H₂O₂ in Opti-MEM for 0, 5, or 10 min, and the resulting lysates were deglycosylated with PNGase F. Immunoblot analysis of 15 μ g of total cell protein was performed with either antibody 3F4 to detect C2 (A), 6H4 to detect C1 (B), or an anti-actin antibody (C). Densitometric analysis was performed on multiple immunoblots, and the results are expressed in terms of pixel intensity of the C2 (D), PrP^C (E), or C1 (F) band at the period of exposure ($n = 3$). ***, $p < 0.001$.

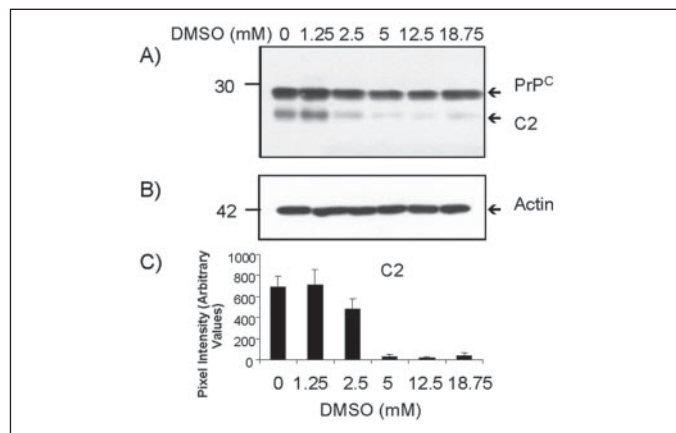
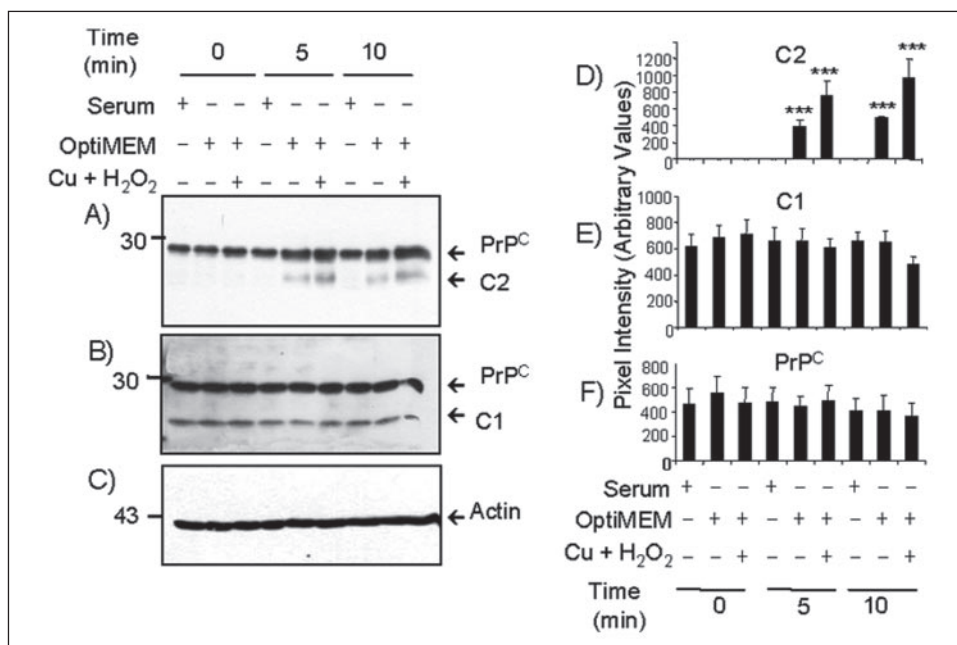


FIGURE 3. Formation of C2 is blocked by the hydroxyl radical scavenger Me₂SO. SH-SY5Y cells expressing wtPrP were exposed to various concentrations of Me₂SO for 5 h in Opti-MEM. Samples (15 μ g of total protein) were digested with PNGase F before immunoblot analysis with antibody 3F4 (A) or an anti-actin antibody (B). C, multiple immunoblots were analyzed by densitometry and expressed as mean pixel intensity for the C2 fragment ($n = 5$).

Failure to Undergo ROS-mediated β -Cleavage Correlates with a Reduced Cell Viability and Increased Levels of Intracellular Free Radicals—In order to determine whether inability to undergo ROS-mediated β -cleavage affected a biological function of PrP^C, we assessed the resistance to oxidative stress of cells expressing PrP Δ oct, PG14, and A116V. Previously, we have shown that SH-SY5Y cells expressing wtPrP have an increased viability, a reduced level of intracellular free radicals, and increased glutathione peroxidase activity as compared with untransfected cells upon exposure to H₂O₂ and Cu²⁺ (17). The viability of cells expressing the various mutants of PrP was assessed by measurement of cell number using Hoescht 33342 staining. Cells expressing PrP Δ oct, PG14, or A116V all displayed significantly reduced viability when challenged with H₂O₂ and Cu²⁺ as compared with cells expressing wtPrP ($p < 0.001$) (Fig. 7A). Measurement of intracellular free radical generation in the cells was made using the fluorescent dye dihydrodichlorofluorescein diacetate in order to ascertain whether radical-mediated cell damage was altered in the cells expressing the PrP mutants. Whereas there was a significant decrease in radical formation

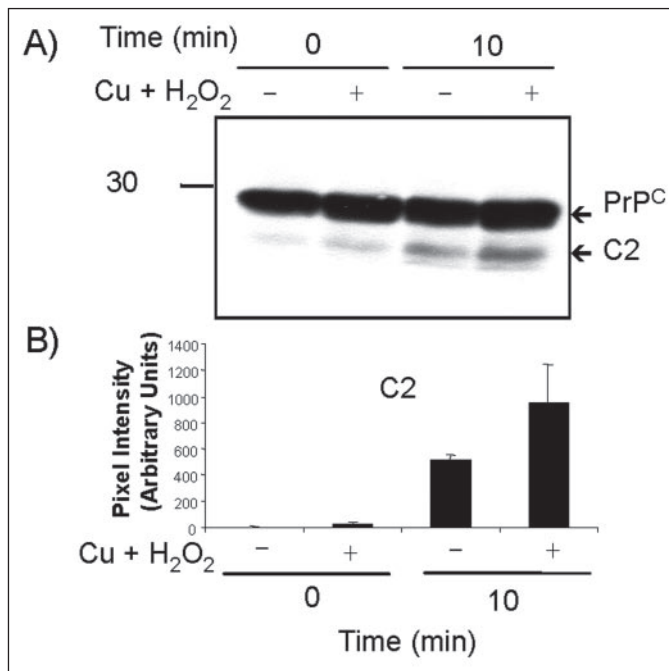


FIGURE 4. C2 is formed at the cell surface. A, SH-SY5Y cells expressing wtPrP were surface-biotinylated for 1 h at 4 °C and then incubated in either Opti-MEM alone or 10 μ M CuSO₄ and 100 μ M H₂O₂ in Opti-MEM. Biotinylated PrP was immunoprecipitated with antibody 3F4 prior to incubation with PNGase F and then immunoblotted with peroxidase-conjugated streptavidin. B, densitometric analysis of multiple immunoblots is shown as pixel intensity of the C2 band ($n = 3$).

in the wtPrP-expressing cells as compared with the untransfected cells, cells expressing PrP Δ oct, PG14, or A116V had a level of radical formation similar to that of the untransfected cells (Fig. 7B). Glutathione peroxidase is a key component of an important antioxidant pathway in neurons, detoxifying H₂O₂ upon glutathione oxidation. The wtPrP-expressing cells had a higher level of glutathione peroxidase activity than the untransfected cells, whereas the cells expressing PrP Δ oct, PG14, or A116V all had significantly reduced glutathione peroxidase activity as compared with wtPrP-expressing cells ($p < 0.001$) (Fig. 7C). Together, these data indicate that cells expressing PrP Δ oct, PG14, or A116V, none

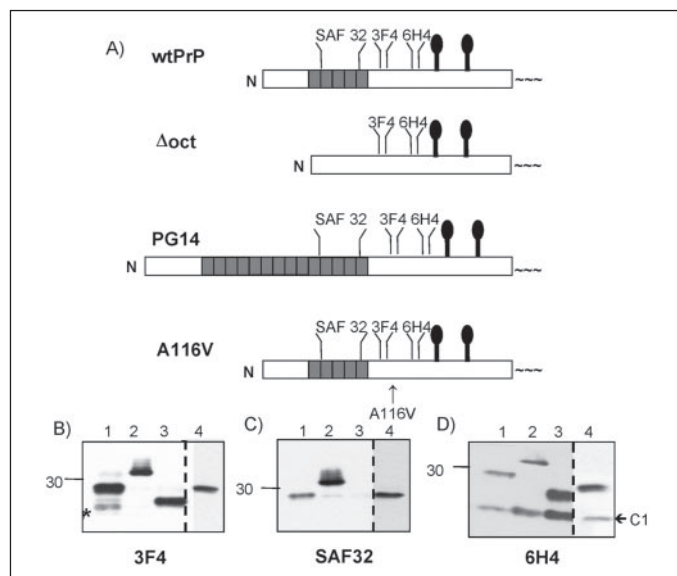


FIGURE 5. Proteolytic processing of PrP^C is altered in PrP mutants. A, schematic diagram of the PrP mutants used. wtPrP is shown as the mature, full-length protein with its C-terminal GPI anchor, two N-linked glycosylation sites (residues 180 and 196, *lollipop*s), and the octapeptide repeat region (*shaded*). PrP^{Δoct} lacks the entire octapeptide region, PG14 has an additional nine octapeptide repeats, and A116V has a single point mutation Ala → Val at position 116. B–D, lysates (15 μg) from SH-SY5Y cells expressing either wtPrP (lane 1), PG14 (lane 2), Δoct (lane 3), or A116V (lane 4) were digested with PNGase F and subjected to immunoblot analysis with 3F4 (B), SAF32 (C), or 6H4 (D). Due to a lower expression level of the A116V construct, 25 μg of protein was loaded, and an increased exposure time was used to detect the protein fragments and is shown by a split in the gel. *, position of C2.

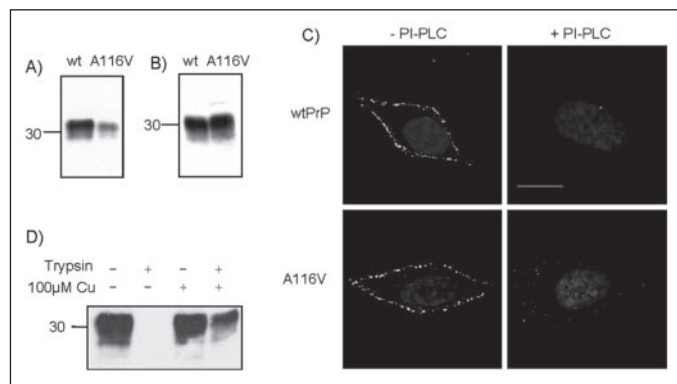


FIGURE 6. A116V is localized at the cell surface and undergoes copper-mediated endocytosis. A, lysates from SH-SY5Y cells (15 μg) expressing either wtPrP or the A116V construct were subjected to immunoblot analysis with 3F4. B, cells expressing either wtPrP or A116V were surface-biotinylated for 1 h at 4 °C, and labeled PrP was immunoprecipitated with 3F4. Biotinylated PrP in the samples was detected by immunoblotting with peroxidase-conjugated streptavidin. C, immunofluorescence microscopy was performed on cells expressing either wtPrP or A116V incubated in the absence or presence of bacterial phosphatidylinositol-specific phospholipase C for 30 min at room temperature. Bar, 10 μm. D, cells expressing A116V were surface-biotinylated for 1 h at 4 °C. The biotinylation reaction was quenched with 50 mM glycine in PBS before incubation of the cells in the presence or absence of 100 μM CuSO₄ delivered as a histidine chelate in Opti-MEM. After 30 min, the samples were scraped into PBS or incubated with trypsin-EDTA to remove cell surface PrP. Lysates were immunoprecipitated, and biotinylated PrP was detected as described above.

of which undergo ROS-mediated β-cleavage, were not protected against oxidative stress in the same way that cells expressing wtPrP were protected.

To further examine the relationship between ROS-mediated β-cleavage and the resistance of cells to oxidative stress, we sought to block β-cleavage of wtPrP and then assess cell viability upon exposure to ROS. We reasoned that antibody SAF32, which binds to the octapeptide repeats may prevent β-cleavage. Cells expressing wtPrP were incubated

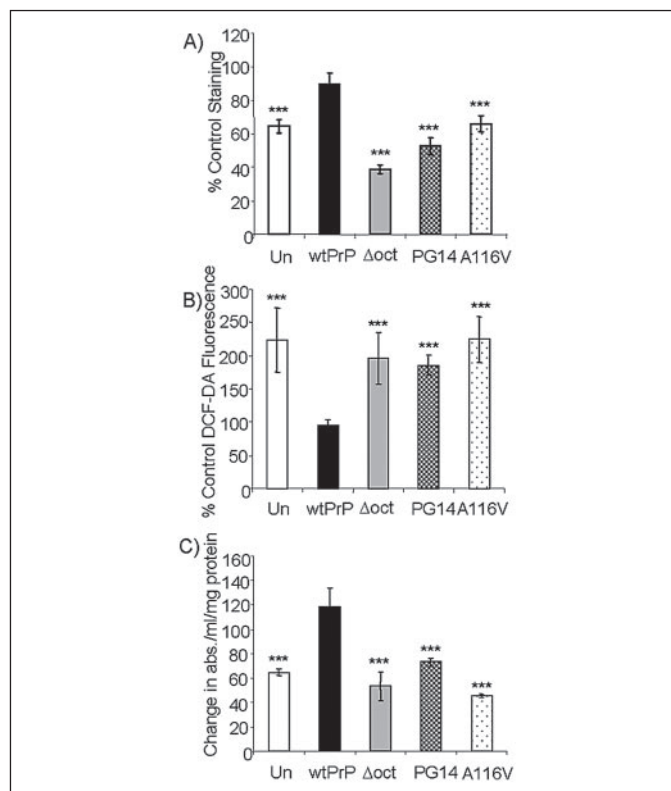


FIGURE 7. Reduced viability and glutathione peroxidase activity and increased radical generation in cells expressing mutants of PrP unable to produce C2. A, Hoescht 33342 staining of untransfected SH-SY5Y (Un) cells or cells containing either wtPrP or the indicated mutants exposed to 8 μM CuSO₄ and 100 μM H₂O₂ for 48 h, expressed as a percentage of control, untreated staining for each individual construct (n = 8). B, measurement of intracellular radical generation using dihydrodichlorofluorescein diacetate in cells treated with 8 μM CuSO₄ and 100 μM H₂O₂ for 4 h. Data are expressed as percentage of control, untreated staining for each individual construct (n = 8). C, glutathione peroxidase activity in untransfected SH-SY5Y cells or cells containing the indicated constructs was determined as described under "Experimental Procedures." Results are shown as change in absorbance/ml/mg protein. ***, p < 0.001.

in the presence of either antibody SAF32 or antibody 3F4 as control prior to exposure to Cu²⁺ and H₂O₂ (Fig. 8). Although the formation of C2 still occurred in cells incubated with 3F4, its production was significantly reduced by SAF32 (Fig. 8, A and C). Furthermore, cells incubated with SAF32 had a significantly lower viability when exposed to Cu²⁺ and H₂O₂ than cells exposed to either 3F4 or no antibody (Fig. 8D), providing further evidence that β-cleavage is involved in the cellular response to oxidative stress.

C2 Formed from the ROS-mediated β-Cleavage of PrP^C Is neither Proteinase K-resistant nor Generated by Calpains—Recently, it has been reported that in scrapie-infected mouse brain and cells, a C2-like fragment is present that is proteinase K-resistant and is generated through cleavage of PrP^{Sc} by calpains (33). In contrast, the C2 fragment generated from wtPrP expressed in the SH-SY5Y cells in the present study was completely proteinase K-sensitive (Fig. 9A). We examined also whether the calpain inhibitor ALLM could block the formation of C2 (Fig. 9, B and D). Interestingly, incubation of the cells with 50 μM ALLM dissolved in Me₂SO led to a reduction in the formation of C2. However, this was due to the free radical quenching effect of Me₂SO, since Me₂SO alone blocked the formation of C2 (Fig. 9, B and D; see Fig. 3), and ALLM dissolved in ethanol did not inhibit the formation of C2, despite the fact that ALLM (at 10 μM) dissolved in either Me₂SO or ethanol completely inhibited the activity of recombinant calpain against a fluorimetric peptide substrate (Fig. 9E). Thus, the calpain inhibitor ALLM failed to block

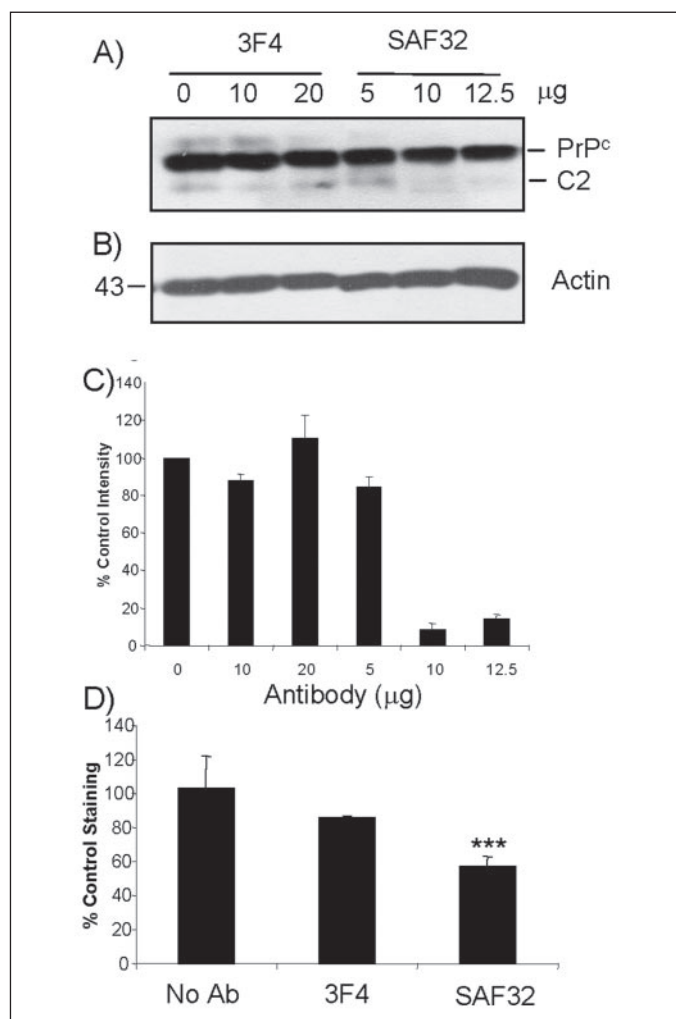


FIGURE 8. Binding of an antibody to the octapeptide repeats blocks β -cleavage and compromises cell viability. A, SH-SY5Y cells expressing wtPrP were incubated with either antibody 3F4 or SAF32 at the indicated concentrations in Opti-MEM for 5 h. The cell lysates were deglycosylated with PNGase F and immunoblotted with either antibody 3F4 to ascertain the C2 levels (A) or an anti-actin antibody (B). C, densitometric analysis of multiple immunoblots is shown as pixel intensity of the C2 band as a percentage of the C2 band produced in the cells not incubated with antibody ($n = 3$). D, Hoechst 33342 staining of SH-SY5Y cells containing wtPrP exposed to 8 μ M CuSO₄ and 100 μ M H₂O₂ for 5 h in the absence or presence of 10 μ g of 3F4 or 10 μ g of SAF32 antibody as indicated. Data are expressed as a percentage of staining in the absence of Cu²⁺ and H₂O₂ for each condition ($n = 8$). ***, $p < 0.001$.

the ROS-mediated β -cleavage of PrP^C in the SH-SY5Y cells, and the resulting C2 fragment was sensitive to proteinase K digestion.

DISCUSSION

Like many proteins, PrP^C is subject to a variety of proteolytic cleavage events that may modulate its biological functions. However, the role of these cleavages and the function of the generated fragments remain to be determined. In the present study, we show for the first time that the ROS-mediated β -cleavage of PrP^C occurs at the cell surface, requires the octapeptide repeat region within PrP^C, and is defective in two forms of PrP associated with prion disease. Crucially, we show that this failure to undergo ROS-mediated β -cleavage correlates with an increased sensitivity of cells to oxidative stress, providing the first direct evidence that β -cleavage may be a critical first step in the mechanism whereby PrP^C protects cells against oxidative stress.

Although the β -cleavage of PrP^C at the C-terminal end of the octapeptide repeats, near residue 90, has been observed by several

groups (18, 21, 24), the mechanism of this cleavage remained elusive until McMahon *et al.* (25) reported that it was ROS-mediated and Cu²⁺-dependent. These authors showed that soluble PrP in conditioned medium underwent β -cleavage upon exposure to millimolar concentrations of H₂O₂ in the presence of 10 μ M Cu²⁺. We have extended this observation to a more *in vivo* setting by showing that exposure of intact cells expressing PrP^C to micromolar concentrations of H₂O₂ and Cu²⁺ stimulates β -cleavage, as evidenced by the formation of the C-terminal fragment C2. It has been hypothesized that PrP^C is involved in the cellular response mechanism to external oxidative stress, possibly acting as a sensor of ROS (34, 35). Although it has previously been shown that ROS causes β -cleavage of soluble PrP (25), it has not been investigated whether the β -cleavage of PrP^C may be involved in the mechanism by which cells respond to oxidative stress. Our observations that surface-biotinylated PrP^C rapidly (within minutes) undergoes β -cleavage upon exposure of cells to ROS and that lack of β -cleavage correlates with an increased sensitivity to oxidative stress provides the first evidence that this processing event is an early step in the cellular response to external oxidative stress.

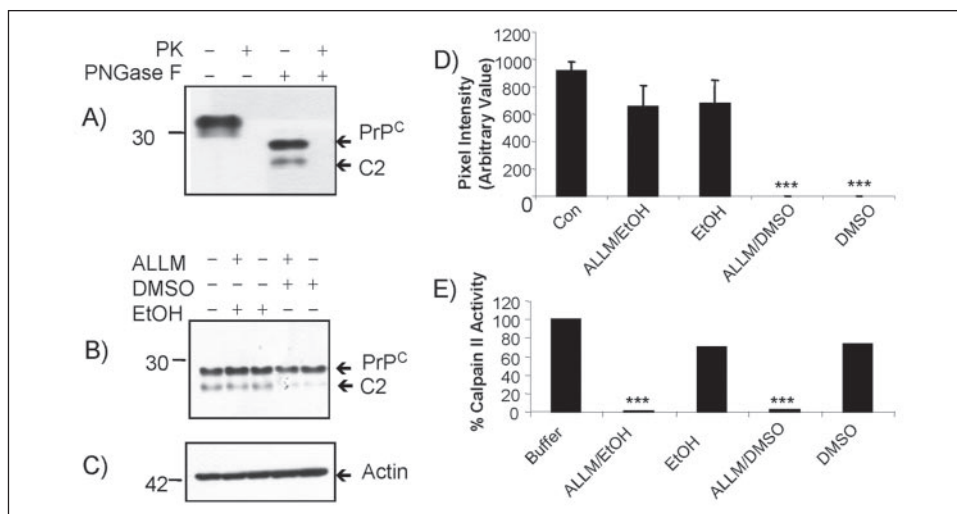
Recently, it has been reported that in scrapie-infected mouse brain and in persistently infected scrapie mouse brain cells, a C2-like fragment is present that is proteinase K-resistant and is generated through cleavage of PrP^{Sc} by calpains (33). The formation of C2 from PrP^C in the SH-SY5Y cells was not blocked by a specific calpain inhibitor, nor was C2 proteinase K resistant, consistent with the study of Yadavalli *et al.* (33) that in uninfected cells and brain tissue the observed C2 fragment is not proteinase K-resistant. Thus, it appears that PrP^C is subject to ROS-mediated β -cleavage, which produces the C2 fragment that is proteinase K-sensitive, whereas PrP^{Sc} is cleaved by calpain to produce a C2-like fragment that is proteinase K-resistant. It is plausible that the conformational change from PrP^C to PrP^{Sc} exposes a cleavage site for calpain that is not accessible in PrP^C.

Through the Fenton reaction, copper (and iron) promote the formation of ROS such as the hydroxyl radical (\cdot OH) from H₂O₂, which, although itself not a ROS, is an important mediator of oxidative stress in neurons (16). When the metal is protein-bound, since the Cu²⁺ ions are in the octapeptide repeats of PrP^C, the oxidative-reduction reaction can locally generate ROS that may react at specific sites in the protein, possibly resulting in peptide bond cleavage (36). The critical involvement of ROS in the β -cleavage of PrP^C is further evidenced by the inhibitory effect of the hydroxyl radical trapping agent Me₂SO. The importance of the Cu²⁺ ions bound at the octapeptide repeats of PrP^C contributing to the ROS-mediated β -cleavage is evidenced by the lack of ROS-mediated cleavage of PrP Δ oct, which lacks the octapeptide repeats and therefore has no Cu²⁺ ions bound in this region of the protein. Although Cu²⁺ binding sites downstream of the octapeptide repeats have been identified (37–39), the Cu²⁺ bound at these sites does not appear to be involved in the ROS-mediated cleavage of PrP^C, as evidenced by the lack of cleavage of PrP Δ oct, which retains these downstream Cu²⁺ binding sites.

Collectively, our data indicate that ROS-mediated β -cleavage of PrP^C may be the first step in a cascade of cellular events that lead the cell to mount a response to increased oxidative stress. Consistent with this is the observation that cells expressing wtPrP have increased viability and glutathione peroxidase activity and reduced intracellular free radicals when exposed to ROS as compared with untransfected cells and that such protective responses to ROS are not observed in the cells expressing PrP Δ oct, which fails to undergo β -cleavage due to the lack of the octapeptide repeats or in cells expressing wtPrP when β -cleavage is blocked by the binding of antibody SAF32 to the octapeptide repeats.

FIGURE 9. C2 is proteinase K sensitive, and its formation is not blocked by calpain inhibitors.

A, lysates (15 μ g of total protein) from cells exposed to 10 μ M CuSO₄ and 100 μ M H₂O₂ were incubated in the presence or absence of PNGase F followed by digestion with 5 μ g/ml proteinase K (PK) and immunoblotted with antibody 3F4. B and C, SH-SY5Y cells expressing wtPrP were incubated in the presence or absence of the calpain inhibitor 50 μ M ALLM for 5 h using either Me₂SO or ethanol as carrier. Samples (15 μ g) were digested with PNGase F before immunoblot analysis using antibody 3F4 (B) or an anti-actin antibody (C). D, densitometric analysis of multiple immunoblots is shown as pixel intensity of the C2 band ($n = 3$). E, recombinant calpain was assayed with a fluorimetric substrate in the absence or presence of 10 μ M ALLM in either Me₂SO or EtOH. Activity is represented as percentage of the activity in the absence of inhibitor and carrier ($n = 2$). ***, $p < 0.001$.



At first sight, it appears somewhat surprising that neither PG14 nor A116V was subject to ROS-mediated β -cleavage. As shown in the present study (for A116V) and elsewhere (for PG14) (12, 32), this is not due to a failure of these mutants of PrP to reach the cell surface, where ROS-mediated β -cleavage occurs. Furthermore, it is not linked to an inability to undergo copper-mediated endocytosis as seen with both PG14 and PrP Δ oct (12), since A116V was efficiently endocytosed upon incubation of the cells with copper. PG14, which contains an extra nine copies of the octapeptide repeat, might, if anything, be expected to be more susceptible to copper-dependent ROS-mediated cleavage. Clearly, this is not the case, and the extended octapeptide repeat region, possibly through the formation of an altered relatively proteinase-resistant structure (40, 41), may somehow prevent ROS-mediated β -cleavage. In the case of A116V, why a single conservative point mutation some 25 residues away from the site of β -cleavage has such a dramatic effect is not immediately obvious. One possibility is that this mutation disrupts the folding of the polypeptide chain and thus prevents ROS-mediated β -cleavage occurring. In this context, it is interesting to note that mutation of Ala¹¹³, Ala¹¹⁵, and Ala¹¹⁸ to valines enhances the folding of peptides spanning this region into compact structural units, significantly enhancing the formation of extensive β -sheet fibrils (42).

The results of the present study do not allow us to directly address which of the proteolytic fragments, N2 or C2, produced from ROS-mediated β -cleavage of PrP^C is responsible for propagating the survival signal. The soluble N2 fragment may act as a signaling molecule analogous to peptide growth factors (34). In support of this is the observation that deletion of the N-terminal residues 23–88 from PrP abrogates the potential to rescue PrP-deficient mice from Doppel-induced neurodegeneration (43) and that cells expressing a construct of PrP in which the N terminus is tethered to the membrane through an uncleaved signal peptide/transmembrane anchor are severely compromised in their resistance to oxidative stress (17). Alternatively, it has been suggested that the GPI-anchored C2 fragment is important in mediating the cellular response to oxidative stress via dimerization and activation of signal transduction pathways (35) and that the protective function of C2 is turned off by subsequent α -cleavage to generate C1 (22). Thus, there is a precursor-product relationship between the C2 and C1 fragments. However, our data would argue against such a relationship, because although PrP Δ oct, PG14, and A116V all failed to produce C2, they all produced amounts of C1 similar to those produced by wtPrP. Rather, we favor the scenario that the α - and β -cleavages of PrP^C are independent

proteolytic events similar to the α - and β -cleavages of the Alzheimer's amyloid precursor protein (44).

The observation that ROS-mediated β -cleavage of PrP^C is defective in the two disease-associated mutants PG14 and A116V adds further weight to the argument that prion diseases are, in part, due to the loss of a normal function of PrP^C (4). Clearly, the inability of cells expressing the disease-associated PG14 and A116V mutants to mount a protective response against oxidative stress would be detrimental. There is increasing evidence that oxidative stress is involved in prion diseases (45–47). This could come about because, upon conversion to PrP^{Sc}, PrP^C is no longer available to be subject to ROS-mediated β -cleavage as part, possibly the first step, of the cellular mechanism to protect against oxidative stress. In cases of prion disease due to mutation in PrP, such as in PG14 and A116V, that prevent ROS-mediated β -cleavage of PrP^C, the normal cellular response to oxidative stress is compromised, and this in turn may contribute to the neurodegeneration observed.

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Reactive Oxygen Species-mediated β -Cleavage of the Prion Protein in the Cellular Response to Oxidative Stress

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