REACTIVE OXYGEN SPECIES-MEDIATED β-CLEAVAGE OF THE PRION PROTEIN IN THE CELLULAR RESPONSE TO OXIDATIVE STRESS

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INTRODUCTION

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) 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].

Amongst the neuroprotective functions of PrP^C are roles in copper homeostasis and the cellular resistance to oxidative stress [6,7]. PrP^C binds Cu^{2+} ions, primarily within the N-terminal octapeptide repeats [8-10], undergoes endocytosis upon exposure of cells to Cu^{2+} [11,12] and modulates neuronal Cu^{2+} 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 (wt) PrP and are more susceptible to oxidative damage and toxicity caused by reactive oxygen species (ROS) [14-17], implicating PrPC in the cellular response to oxidative stress. However, the mechanism by which PrPC mediates this protective effect is not known.

PrPC is a glycosyl-phosphatidylinositol (GPI) anchored glycoprotein that undergoes a variety of proteolytic processing events. The protein can be cleaved at amino acids 110/111 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]. PrPC 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 PrPC is proteolytically shed from the cell surface by a zinc metalloprotease that has similar properties to the α-secretase cleavage of the Alzheimer’s 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 PrPC 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, 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 (PrPΔoct) 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 PrPC protects cells against oxidative stress.

**EXPERIMENTAL PROCEDURES**

**cDNA constructs and cell culture**

The construction of wtPrP, PrPΔoct and PG14 in pIREsneo has been described previously [12]. A116V was generated from wtPrP using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) with the following primers: sense 5’-

CAGGGGCTGCGTAGCTGGGGCAGTAG

and the resulting construct verified by DNA sequencing. Human neuroblastoma SH-SY5Y cells were cultured, transfected by electroporation and pooled, stable cell lines obtained by antibiotic selection as described previously [27]. Copper was routinely administered to the cells as CuSO4 in the presence of fetal calf serum (FCS) to provide a source of both albumin and histidine for the Cu2+ to complex to. When the cells had reached confluency, the monolayer was washed twice with electroporation- and pooled, stable cell lines obtained by antibiotic selection as described previously [27]. Copper was routinely administered to the cells as CuSO4 in the presence of fetal calf serum (FCS) to provide a source of both albumin and histidine for the Cu2+ to complex to. When the cells had reached confluency, the monolayer was washed twice with OptiMEM before incubation with the relevant compounds for the specified periods of exposure in OptiMEM. Cells were harvested into phosphate-buffered saline (PBS; 1.5 mM KH2PO4, 2.7 mM Na2HPO4, 150 mM NaCl, pH 7.4) pelleted by centrifugation at 1000 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 cocktail). The protein content of each lysate was determined using bicinchoninic acid in a microtitre plate assay with bovine serum albumin as standard [28].

**Surface biotinylation and immunoprecipitation**

Cells at confluence were incubated for 1 h at 4°C with 0.5 mg/ml Biotin sulfo-NHS, washed 3 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 μM CuSO4 and 100 μM H2O2 in OptiMEM. 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 g and the supernatant 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 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-labelled cells were incubated for 30 min at 37°C in the absence or presence of 100 µM CuSO4 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 5µg/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 poly(vinylidene) difluoride membrane (Amersham, Little Chalfont, U.K.). 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 visualised using an enhanced chemiluminescence detection system (Amersham).

**Recombinant calpain activity assay**

The activity of 20 nM recombinant Calpain-2 (Calbiochem) was measured using 5 µM (FAM)-Gly-Gly-Gly-Gln-Leu-Tyr-Gly-Gly(DPA)-Arg-Arg-Lys(TAMRA)NH2 (a gift from GlaxoSmithKline, Harlow, UK) in 60 mM imidazole/HCl, 5 mM L-Cysteine, 2.5 mM glutathione (reduced) and 5 mM CaCl2, pH 7.3. The broad spectrum calpain inhibitor N-Acetyl-Leu-Leu-Met-CHO (ALLM) resuspended in either DMSO 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 was 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 was monitored by pre-labelling 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 µg/ml Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) 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) for 1 h and mounted on slides using fluoromount G mounting medium (SouthernBiotech). Individual cells were visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision Inc., USA). Data was collected from 30-40 0.1 µm thick optical sections, and 3-D datasets were deconvolved using the softWoRx programme (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 x 10^4 per 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 either H2O2 (100 µM), CuSO4 (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 stained with the DNA-binding fluorochrome Hoechst 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 H₂O₂ (100 μM), CuSO₄ (8 μM) or both reagents in 5% FCS-containing medium for 6 h using 100 μM dihydrodichlorofluorescein diacetate (DCF-DA) as described previously [17]. Glutathione peroxidase activity was measured using 50 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 mM glutathione and 1 U/ml glutathione reductase at room temperature upon the addition of 0.1 ml cumene hydroperoxide (1.5 mM) as described previously [17].

**Statistical analysis**

All analyses were subject to Kruskal-Wallis non-parametric 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<sup>C</sup> in SH-SY5Y cells is subject to α- and β-cleavages**

The proteolytic processing of murine PrP<sup>C</sup> containing the 3F4 epitope (wtPrP) stably expressed in the human neuroblastoma SH-SY5Y cell line was examined using antibodies that recognise 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 recognises 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 recognises the engineered epitope MHKM (residues 108-111 of murine PrP), detected both full-length PrP and the C2 fragment of molecular weight 21 kDa but not the C1 fragment as α-cleavage destroys the epitope recognised by this antibody (Fig. 1C) [20,21]. Antibody 6H4, which recognises an epitope in the C-terminal half of the protein (residues 144-152), detected full-length PrP, C2 and the C1 fragment of molecular weight 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<sup>C</sup> 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<sup>C</sup> is upregulated when cells are subjected to oxidative stress**

As there is some evidence to suggest that β-cleavage of PrP<sup>C</sup> 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 OptiMEM caused an increase in the production of C2 (Fig. 2A and D) due to the removal of survival factors on withdrawal of the serum from the medium [15]. In the presence of 100 μM H₂O₂ and 10 μM 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. 2A and D). In contrast to the changes in C2, the levels of neither full-length PrP (Fig. 2B and E) nor the C1 fragment (Fig. 2B 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<sup>C</sup> is indeed a ROS-mediated event, the effect of the hydroxyl radical quencher DMSO was examined (Fig. 3). When cells expressing wtPrP were incubated in serum-free OptiMEM in the presence of DMSO, there was a dose-dependent reduction in the production of C2.
(Fig. 3A 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 µM H\(_2\)O\(_2\) and 10 µM Cu\(^{2+}\) (Fig. 4). Following immunoprecipitation of PrP with antibody 3F4, biotinylated full-length PrP and C2 were visualised 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 OptiMEM for 10 min, and this was further increased upon treatment of the cells with H\(_2\)O\(_2\) and Cu\(^{2+}\) 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\(^{\Delta oct}\) that lacks the copper-binding octapeptide repeat region [12] (Fig. 5A). Lysates from cells expressing PrP\(^{\Delta oct}\) were subjected to immunoblot analysis with antibodies SAF32, 3F4 and 6H4 (Fig. 5B-D). SAF32 failed to detect PrP\(^{\Delta oct}\) as 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\(^{\Delta oct}\), 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 9 copies of the octapeptide repeat and is associated with familial human prion disease [29,30] and A116V in which Ala 116 (murine PrP numbering, equivalent to Ala 117 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. 5B-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 antibodies 3F4 or 6H4 even after prolonged exposure of the immunoblots (Fig. 5B and D). Even upon treatment of the cells expressing PG14 or A116V with H\(_2\)O\(_2\) and Cu\(^{2+}\) 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 localised 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 localisation 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 PI-PLC which cleaves the GPI anchor releasing the protein from the membrane. As neither the PrP\(^{\Delta oct}\) nor the PG14 mutants are endocytosed when cells are exposed to Cu\(^{2+}\) 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 Cu\(^{2+}\) ions sufficient to promote endocytosis of wtPrP [12], this mutant was rapidly endocytosed (Fig. 6D). Thus, 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.

**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\(^\text{C}\), we assessed the resistance to oxidative stress of cells expressing PrP\(^{\Delta\text{oct}}\), 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 H\(_2\)O\(_2\) and Cu\(^{2+}\) [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\(^{\Delta\text{oct}}\), PG14 or A116V all displayed significantly reduced viability when challenged with H\(_2\)O\(_2\) and Cu\(^{2+}\) as compared to cells expressing wtPrP (p < 0.001) (Fig. 7A). Measurement of intracellular free radical generation in the cells was made using the fluorescent dye DCF-DA 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 in the wtPrP expressing cells as compared to the untransfected cells, cells expressing PrP\(^{\Delta\text{oct}}\), PG14 or A116V had a similar level of radical formation as the untransfected cells (Fig. 7B). Glutathione peroxidase is a key component of an important antioxidant pathway in neurons, detoxifying H\(_2\)O\(_2\) upon glutathione oxidation. The wtPrP expressing cells had a higher level of glutathione peroxidase activity than the untransfected cells, whereas the cells expressing PrP\(^{\Delta\text{oct}}\), PG14 or A116V all had significantly reduced glutathione peroxidase activity as compared to wtPrP expressing cells (p < 0.001) (Fig. 7C). Together these data indicate that cells expressing PrP\(^{\Delta\text{oct}}\), PG14 or A116V, none 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 in the presence of either antibody SAF32 or antibody 3F4 as control prior to exposure to Cu\(^{2+}\) and H\(_2\)O\(_2\) (Fig. 8). Although the formation of C2 still occurred in cells incubated with 3F4, its production was significantly reduced by SAF32 (Fig. 8A and C). Furthermore, cells incubated with SAF32 had a significantly lower viability when exposed to Cu\(^{2+}\) and H\(_2\)O\(_2\) 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\(^\text{C}\) 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\(^{\text{Sc}}\) 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. 9B and D). Interestingly, incubation of the cells with 50 µM ALLM dissolved in DMSO led to a reduction in the formation of C2. However, this was due to the free radical quenching effect of DMSO, as DMSO alone blocked the formation of C2 (Fig. 9B and D and 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 DMSO or ethanol completely inhibited the activity of recombinant calpain against a fluorimetric peptide substrate (Fig. 9E). Thus the calpain inhibitor ALLM failed to block the ROS-mediated β-cleavage of PrP\(^{\text{C}}\) in the SH-SY5Y cells and the resulting C2 fragment was sensitive to proteinase K digestion.
DISCUSSION

Like many proteins PrPC 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 PrPC occurs at the cell surface, requires the octapeptide repeat region within PrP 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 PrPC protects cells against oxidative stress.

Although the β-cleavage of PrPC 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 mM concentrations of H2O2 in the presence of 10 µM Cu2+. We have extended this observation to a more in vivo setting by showing that exposure of intact cells expressing PrPC to µM concentrations of H2O2 and Cu2+ stimulates β-cleavage as evidenced by the formation of the C-terminal fragment C2. It has been hypothesised that PrPC 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 PrPC may be involved in the mechanism by which cells respond to oxidative stress. Our observations that surface biotinylated PrPC 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 PrPSc by calpains [33]. The formation of C2 from PrPC 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 PrPC is subject to ROS-mediated β-cleavage which produces the C2 fragment that is proteinase K sensitive, while PrPSc is cleaved by calpain to produce a C2-like fragment that is proteinase K resistant. It is plausible that the conformational change from PrPC to PrPSc exposes a cleavage site for calpain that is not accessible in PrPC.

Through the Fenton reaction copper (and iron) promote the formation of ROS such as the hydroxyl radical (·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, as the Cu2+ ions are in the octapeptide repeats of PrPC, 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 PrPC is further evidenced by the inhibitory effect of the hydroxyl radical trapping agent DMSO. The importance of the Cu2+ ions bound at the octapeptide repeats of PrPC contributing to the ROS-mediated β-cleavage is evidenced by the lack of ROS-mediated cleavage of PrPΔoct 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 PrPC as evidenced by the lack of cleavage of PrPΔoct which retains these downstream Cu2+ binding sites.

Collectively our data indicate that ROS-mediated β-cleavage of PrPC 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 to untransfected cells, and that such protective responses to ROS are not observed in the cells expressing PrP\(\Delta\)oct which fails to undergo \(\beta\)-cleavage due to the lack of the octapeptide repeats or in cells expressing wtPrP when \(\beta\)-cleavage is blocked by the binding of antibody SAF32 to the octapeptide repeats.

At first sight it appears somewhat surprising that neither PG14 nor A116V were subject to ROS-mediated \(\beta\)-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 \(\beta\)-cleavage occurs. Furthermore, it is not linked to an inability to undergo copper-mediated endocytosis as seen with both PG14 and PrP\(\Delta\)oct [12], as A116V was efficiently endocytosed on incubation of the cells with copper. PG14, which contains an extra 9 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 \(\beta\)-cleavage. In the case of A116V why a single conservative point mutation some 25 residues away from the site of \(\beta\)-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 \(\beta\)-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 \(\beta\)-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 \(\beta\)-cleavage of PrP\(^C\) is responsible for propagating the survival signal. The soluble N2 fragment may act as a signalling 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 dimerisation and activation of signal transduction pathways [35] and that the protective function of C2 is turned off by subsequent \(\alpha\)-cleavage to generate C1 [22].

The observation that ROS-mediated \(\beta\)-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 on conversion to PrP\(^{Sc}\), PrP\(^C\) is no longer available to be subject to ROS-mediated \(\beta\)-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 \(\beta\)-cleavage of PrP\(^C\), the normal cellular response to oxidative stress is
compromised and this in turn may contribute to the neurodegeneration observed.

References


Footnotes

This work was supported by the Medical Research Council of Great Britain, the European Union (QLG3-CT-2001-02353) and the Wellcome Trust (Bioimaging Facility, University of Leeds). DAT was in receipt of a Biotechnology and Biological Sciences Research Council studentship. WSSP was in receipt of an Emma and Leslie Reid studentship from the University of Leeds.

Abbreviations used: ADAM, a disintegrin and metalloprotease; ALLM, N-Acetyl-Leu-Leu-Met-CHO; DCF-DA, dihydrodichlorofluorescein diacetate; DMSO, dimethyl sulphoxide; FCS, fetal calf serum; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, Peptide:N-glycosidase F; PrPC, cellular form of the prion protein; PrPSc, infectious, protease resistant form of PrP; ROS, reactive oxygen species.

Figure Legends

**Figure 1. PrPC in SH-SY5Y cells is subject to both α- and β-cleavages.**

(A) Schematic diagram of the proteolysis of PrPα and the epitopes recognised by the antibodies used in this study. Mature, full-length PrPC is shown with its C-terminal GPI anchor, two N-linked glycosylation

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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 recognised by both 3F4 and 6H4, whereas C1 is only recognised by 6H4. (B-D) Lysates (15µg) from SH-SY5Y cells expressing wtPrP were incubated in the absence or presence of PNGase F and immunoblotted with (B) SAF32, (C) 3F4 and (D) 6H4 (25µg of lysate). Molecular weight markers (kDa) are indicated on the left.

Figure 2. Copper and H2O2 upregulate the formation of C2.
SH-SY5Y cells stably expressing wtPrP were either maintained in 10% FCS-containing DMEM (Serum), maintained in OptiMEM medium or treated with 10 µM CuSO4 and 100 µM H2O2 in OptiMEM for 0, 5 or 10 min and the resulting lysates deglycosylated with PNGaseF. Immunoblot analysis of 15µg 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 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 DMSO.
SH-SY5Y cells expressing wtPrP were exposed to various concentrations of DMSO for 5 h in OptiMEM. Samples (15µg total protein) were digested with PNGaseF before immunoblot analysis with antibody 3F4 (A) or an anti-actin antibody (B). (C) Multiple immunoblots were analysed by densitometry and expressed as mean pixel intensity for the C2 fragment (n = 5).

Figure 4. C2 is formed at the cell surface.
(A) SH-SY5Y cells expressing wtPrP were surface biotinylated for 1h at 4°C and then incubated in either OptiMEM alone or 10 µM CuSO4 and 100 µM H2O2 in OptiMEM. 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).

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, lollipops) and the octapeptide repeat region (shaded). PrPΔoct lacks the entire octapeptide region, PG14 has an additional 9 octapeptide repeats and A116V has a single point mutation Ala→Val at position 116. (B-D) Lysates (15µg) from SH-SY5Y cells expressing either wtPrP (lane 1), PG14 (lane 2), Δoct (lane 3) or A116V (lane 4) were digested with PNGaseF and subjected to immunoblot analysis with 3F4 (B), SAF 32 (C) or 6H4 (D). Due to lower expression level of the A116V construct, 25 µg protein was loaded and an increased exposure time was used to detect the protein fragments and is shown by a split in the gel. * indicates the position of C2.

Figure 6. A116V is localised at the cell surface and undergoes copper-mediated endocytosis.
(A) Lysates from SH-SY5Y cells (15µg) 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 labelled PrP 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 PI-PLC for 30 min at room temperature. Bar = 10µm. (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
OptiMEM. 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 detected as described above.

**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 DCF-DA in cells treated with 8 µM CuSO₄ and 100 µM H₂O₂ for 4 h. Data 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 in the Experimental section. Results are shown as change in absorbance/ml/mg protein. *** p < 0.001.

**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 OptiMEM for 5 h. The cell lysates were deglycosylated with PNGaseF and immunoblotted with either (A) antibody 3F4 to ascertain the C2 levels or (B) an anti-actin antibody. (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) Hoescht 33342 staining of SH-SY5Y cells containing wtPrP exposed to 8 µM CuSO₄ and 100 µM H₂O₂ for 5 h in the absence or presence of 10 µg 3F4 or 10 µg SAF32 antibody as indicated. Data expressed as a percentage of staining in the absence of Cu²⁺ and H₂O₂ for each condition (n = 8). *** p < 0.001.

**Figure 9. C2 is proteinase K sensitive and its formation is not blocked by calpain inhibitors.**

(A) Lysates (15µg total protein) from cells exposed to 10 µM CuSO₄ and 100 µM H₂O₂ were incubated in the presence or absence of PNGase F followed by digestion with 5µg/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 µM ALLM for 5 h using either DMSO or ethanol as carrier. Samples (15µg) were digested with PNGaseF 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 µM ALLM in either DMSO or EtOH. Activity is represented as percentage of the activity in the absence of inhibitor and carrier (n = 2). *** p < 0.001.
Figure 1

A) 

\[ \text{PrP}^C \quad \text{N} \quad \begin{array}{c} \text{SAF32} \\ 3F4 \\ 6H4 \end{array} \quad \sim \sim \quad \text{GPI} \]

\[ \beta\text{-cleavage} \quad \text{ROS?} \quad \alpha\text{-cleavage} \quad \text{ADAM?} \]

\[ \text{N2} \quad \text{C2} \quad \text{N1} \quad \text{C1} \]

B) SAF32

C) 3F4

D) 6H4
Figure 2

A) 30

B) 30

C) 43

D) 0 5 10

E) 0 5 10

F) 0 5 10

---

Table:

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<tr>
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Graphs:

- **D)**
  - C2
  - C1

- **E)**
  - PrPC

- **F)**
  - Actin

Legend:

- Serum
- OptiMEM
- Cu + H₂O₂

---

Legend:

- A)
- B)
- C)

---

Notes:

- PrPC
- Actin

---

References:

- C2
- C1

---

Additional Information:

- Pixel Intensity (Arbitrary Values)

- Serum
- OptiMEM
- Cu + H₂O₂

---

Figure 2

- A) 30
- B) 30
- C) 43
- D) 0 5 10
- E) 0 5 10
- F) 0 5 10

---

Legend:

- A)
- B)
- C)
Figure 3

A) DMSO (mM) 0 1.25 2.5 5 12.5 18.75

B) Actin

C) Pixel Intensity (Arbitrary Values)

DMSO (mM) 0 1.25 2.5 5 12.5 18.75
Figure 4

A) Time (min) 0 10
Cu + H₂O₂ - + - +

B) Pixel Intensity (A.U.)
Cu + H₂O₂ 0 10 - + - +
Time (min)
Figure 5

A) wtPrP

\[\begin{array}{c}
\text{N} \\
\end{array}\]

Δoct

\[\begin{array}{c}
\text{N} \\
\end{array}\]

PG14

\[\begin{array}{c}
\text{N} \\
\end{array}\]

A116V

\[\begin{array}{c}
\text{N} \\
\end{array}\]

B) 3F4

\[\begin{array}{cccc}
1 & 2 & 3 & 4 \\
30 &  &  & \\
\end{array}\]

C) SAF32

\[\begin{array}{cccc}
1 & 2 & 3 & 4 \\
30 &  &  & \\
\end{array}\]

D) 6H4

\[\begin{array}{cccc}
1 & 2 & 3 & 4 \\
30 &  &  & \\
\end{array}\]
Figure 8

A) 3F4  SAF32

0  10  20  5  10  12.5 µg

B) Actin

43

C) % Control Intensity

D) Antibody (µg)

% Control Staining

No Ab  3F4  SAF32

***
Figure 9

A) PK - + - +  
PNGase F - - + +  

B)  
ALLM - + - + -  
DMSO - - + + +  
EtOH - + + - -  

C)  

D)  
% Calpain II Activity  

E)  
Pixel Intensity (Arbitrary Value)
Reactive oxygen species-mediated β-cleavage of the prion protein in the cellular response to oxidative stress
Nicole T. Watt, David R. Taylor, Andrew Gillott, Daniel A. Thomas, W. Sumudhu S. Perera and Nigel M. Hooper

J. Biol. Chem. published online August 24, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M507327200

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