Endogenous Proteolytic Cleavage of Disease-associated Prion Protein to Produce C2 Fragments Is Strongly Cell- and Tissue-dependent* [S]

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The abnormally folded form of the prion protein (PrPSc) accumulating in nervous and lymphoid tissues of prion-infected individuals can be naturally cleaved to generate a N-terminal-truncated fragment called C2. Information about the identity of the cellular proteases involved in this process and its possible role in prion biology has remained limited and controversial. We investigated PrPSc N-terminal trimming in different cell lines and primary cultured nerve cells, and in the brain and spleen tissue from transgenic mice infected by ovine and mouse prions. We found the following: (i) the full-length to C2 ratio varies considerably depending on the infected cell or tissue. Thus, in primary neurons and brain tissue, PrPSc accumulated predominantly as untrimmed species, whereas efficient trimming occurred in Rov and MovS cells, and in spleen tissue. (ii) Although C2 is generally considered to be the counterpart of the PrPSc proteinase K-resistant core, the N termini of the fragments cleaved in vivo and in vitro can actually differ, as evidenced by a different reactivity toward the Pc248 anti-octarepeat antibody. (iii) In lysosome-impaired cells, the ratio of full-length versus C2 species dramatically increased, yet efficient prion propagation could occur. Moreover, cathepsin but not calpain inhibitors markedly inhibited C2 formation, and in vitro cleavage by cathepsins B and L produced PrPSc fragments lacking the Pc248 epitope, strongly arguing for the primary involvement of acidic hydrolases of the endolysosomal compartment. These findings have implications on the molecular analysis of PrPSc and cell pathogenesis of prion infection.

Prions are the infectious agent of transmissible spongiform encephalopathies (TSE), a group of fatal neurodegenerative disorders that include scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jakob disease in humans. The pathogenesis of these diseases is crucially linked to the cellular prion protein (PrPc) (1), a host-encoded glycoprotein attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor, whose normal function is uncertain (2). Infected individuals accumulate an abnormal form of this protein (PrPSc), principally in their nervous and lymphoid tissues (3). Conversion of PrPc into PrPSc seems to take place at the cell surface or along the endocytic pathway. It involves a profound conformational change, leading to the acquisition of new properties such as insolubility in non-denaturing detergent, a strong tendency to aggregate, and an increased resistance to protease digestion, properties that are commonly used to distinguish the two PrP isoforms. Incubation of TSE-infected tissue homogenate with proteinase K (PK) in conditions that completely degrade PrPc generates N-terminal-truncated fragments of PrPSc, referred to as PrPres. Molecular analysis of the relative mass and glycoform ratio of these fragments allows the categorization of the clinicopathological heterogeneous TSE affecting humans and animals (4, 5). This phenotypic diversity is due to the existence of multiple strains of prion in conjunction with host genetic factors including prnp gene polymorphism or mutation. Variation in structural organization of PrPSc within multimers is thought to underlie prion strain diversity. These strain-specific, conformational differences in turn lead to exposure of distinct cleavage sites for PK.

Proteolytic processing of PrPSc has been shown to occur both in brain tissue and cultured cells. A well recognized event is N-terminal truncation leading to the production of PrPSc species commonly referred to as C2, possibly a step toward its complete degradation. Cleavage to produce C2 takes place within the unstructured region of the molecule, distal to the tandem array of octarepeats, and upstream of the physiological cleavage site of PrPc (position 111–112, human numbering) leading to a fragment called C1 (6). C2 is PK resistant and assumed to be the in vivo counterpart of the C-proximal fragment generated by PK digestion of full-length PrPSc. The presence of the N-terminal-truncated PrPSc species in infected brain tissue has been reported in naturally affected species, humans and sheep (6–9), as well as in mouse and hamster models (10–14). Immunochemical studies in sheep combined with PrP peptide mapping have demonstrated the intracellular accumulation in the brain and lymphoid tissues of the various N-terminal-truncated PrPSc species, some of which might correspond to C2 (15, 16). More recently, a region-specific deposition of C2 fragments was reported in sheep brain (9). Altogether these findings provide some evidence that the endocel-
lular processing of PrPSc can be influenced by the agent strain but also possibly by the cell or tissue where it propagates.

Cell culture systems steadily infected with prions offer a convenient system in which PrPSc processing can be studied. N-terminal-truncated, C2-like fragments present before any PK digestion have been observed to accumulate in several mouse cell models, including N2a, GT1, and SMB cell lines (12, 13, 17). This trimming can occur within a few hours after PrPSc acquires its protease resistance, as revealed by the use of metabolically labeled or more recently, tetracysteine-tagged PrP (12, 18). Although matrix metalloproteases have been ascribed a role in the generation of the C1 PrPc fragment (19), the identity and relative contribution of the cellular proteases acting in PrPSc processing is less clear. Treatments of cultures by lysosomotropic compounds such as NH4Cl have been reported to inhibit the generation of C2 cleavage products (12, 17), thus potentially involving hydrolases from the acidic endosomal cell compartment, a recognized site of PrPSc accumulation (20–22). Cysteine protease inhibitors have also been shown to affect PrPSc clearance in cell culture (23). One detailed study has led to the proposal that endoproteolytic C2 cleavage of PrPSc, and prion propagation, are calpain-dependent processes (13). Although lysosome inhibition appeared to prevent PrPSc trimming without any major effect on its biosynthesis (12, 17), cysteine protease inhibitors were shown to either increase (23), reduce (24) the PrPSc steady-state level, or leave it unaffected (18) depending on the cell model, thus raising the possibility that cysteine proteases may indirectly control PrPSc propagation.

In this study, we investigated the endogenous processing of PrPSc in various cell cultures and mouse tissues infected by the same TSE agent. We found that the proportion of N-terminal-truncated versus full-length molecules varies considerably depending on the cellular environment. This process, in which hydrolases from the acidic cell compartment, not calpain, appeared to be primarily involved, did not or only marginally affect prion formation in the cell culture. We also show that the N terminus of naturally trimmed PrPSc molecules can differ from those produced by PK digestion. Our findings bring new information on the natural processing of PrPSc molecules, which is important for prion cell biology and molecular characterization or subtyping of TSE agents.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rov cells (clone Rov9) and Rom cells are derived from the RK13 epithelial cell line and express the ovine or mouse PrP, respectively, in a doxycycline-dependent manner (25, 26). They were grown in Opti-MEM medium supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin, and split at a 1/4 dilution once a week. MovS cells (clone MovS6) are Schwann cell-derived, immortalized cells isolated from tg338 mice constitutively expressing the VRQ allele of ovine PrP (27). The cells were grown in Opti-MEM medium supplemented with 10% FCS plus antibiotics and split once a week at 1/10 dilution. CAD cells are issued from a clone of the CathA cell line, originally derived from mouse brain neurons (28). The cells were cultivated in Opti-MEM (Invitrogen) supplemented with 10% FCS, and split after mechanical resuspen-

**PrPSc Trimming Variation in Cell and Tissue Types**

| Experimental Procedure | Description |
|------------------------|-------------|
| **Cell Culture**—Rov cells (clone Rov9) and Rom cells | Derived from RK13 epithelial cell line and express the ovine or mouse PrP, respectively, in a doxycycline-dependent manner. |
**PrPSc Trimming Variation in Cell and Tissue Types**

centrifugation at 1000 \( \times g \) for 2 min before use or freezing. For analysis, brain and spleen homogenates were diluted in TL1 or TNT buffers. The protein concentration of cells and tissue lysates was determined by BCA (Pierce). The tissues were frozen immediately after removal to minimize spontaneous proteolysis and precautions were taken during the preparation of lysates.

**PrPSc Sedimentation Experiment**—250 to 500 \( \mu g \) of protein from cell or tissue lysates of control or infected samples were sedimented at 22,000 \( \times g \) for 30 min. Supernatants were recovered and pellets were washed once with TL1 buffer and resolubilized in Laemmli sample buffer for SDS-PAGE analysis. In some experiments, pellets were further resuspended in TL1 buffer and treated with PK (see below) before denaturation with Laemmli sample buffer.

**Cu\(^{2+}\)-IMAC Hi-Trap Chromatography**—The AKTA Purification FPLC chromatographic system was used (GE Healthcare). A 1-ml Hi-Trap IMAC column (GE Healthcare) was charged with 0.2 M CuSO\(_4\). The column was equilibrated with TNT buffer containing 3 mM imidazole. One milliliter of cell (1 mg of protein) or 0.5 ml of brain lysates (0.75 mg of protein) were injected into the column. The flow-through fraction was recovered, the column was washed, and a 10-min linear gradient of 3–200 mM imidazole in TNT buffer was applied to elute column-bound proteins at a flow rate of 1 ml/min; 0.5-ml fractions were collected. As a final step, 6 \( \mu l \) urea was used to remove tightly bound proteins. Column fractions were analyzed by Western blotting for PrP detection before and after PK treatment. Individual columns were dedicated to each type of lysate.

**Proteinase K Digestion**—Aliquots of 50 \( \mu l \) of whole cell lysates were treated with 10 \( \mu g/ml \) (10 \( \mu g/mg \) of protein) of PK at 37 °C for 1 h and denatured with Laemmli sample buffer. Mouse brain and spleen lysates were either treated with 50 \( \mu g/ml \) (10 \( \mu g/mg \) of protein) of PK at 37 °C for 1 h or PK digested according to the Bio-Rad test protocol (39). To analyze cell lysates fractionated on IMAC columns, 400 \( \mu l \) of eluted fractions were treated with 5 \( \mu g/ml \) of PK and then precipitated with cold methanol after addition of 20 \( \mu g \) of bovine serum albumin as a protein carrier. Methanol-precipitated proteins were sedimented at 22,000 \( \times g \) for 15 min and the pellets were solubilized in 40 \( \mu l \) of 1× sample buffer. To analyze brain lysates, 50 \( \mu l \) of IMAC column fractions were digested with 10 \( \mu g/ml \) of PK for 1 h at 37 °C and then denatured with 4× sample buffer.

**Thromolysin Treatment**—Cell and brain lysates were treated with thromolysin (Sigma) as previously described (40) with slight modifications. Thromolysin was used at a concentration of 10 \( \mu g/ml \) (10 \( \mu g/mg \) protein) for cell lines and CGN, and at 200 \( \mu g/ml \) for brain lysates. After incubation at 37 °C for 1 h, samples were denatured with an equal volume of 2× sample buffer.

**Cathepsins Treatment**—Sedimentation pellets of infected Rov9 cell lysates were solubilized in cathepsin digestion buffer containing (i) dibasic sodium phosphate brought to pH 6 or 5 with citric acid; (ii) L-cysteine (18 or 195 mM) for redox conditions mimicking that in slightly acidic or acidic compartments, respectively (41). Human liver cathepsin B and L (Merck) were used at 50 \( \mu g/ml \) overnight at 37 °C. Reactions were terminated by addition of an equal volume of 2× sample buffer.

**Quantification of Deglycosylated PrPSc Species**—Samples were treated with PNGase F according to the manufacturer’s instructions (New England Biolabs) and quantification of full-length and C2 PrPSc bands was determined by GeneTools software after acquisition of chemiluminescent signals with a GeneGnome digital imager (Syngene).

**Removal of GPI Anchor**—Removal of the GPI anchor with hydrofluoric acid was performed according to a published procedure (42) with one critical modification. Briefly, 1 volume of crude lysates from uninfected brain or cell materials was mixed directly with 6 volumes of aqueous 48% hydrofluoric acid (Merck), incubated for 24 h at 4 °C, and then vacuum-dried material was solubilized in 2× SDS-PAGE sample buffer. PrPSc was immunodetected using ICSM4 mAb, specific for the unglycosylated form.

**Immunoblotting**—Either 12 or 4–12% NuPage gels (Invitrogen) were used for SDS-PAGE. Transfer of proteins on nitrocellulose filters was performed using a semi-dry transblot system (Bio-Rad). For detection of PrP by immunoblotting, an enhanced chemiluminescence (ECL) detection system (Pierce or Roche) was used with goat anti-mouse IgG coupled to peroxidase as secondary antibodies.

**RESULTS**

**PrPSc Processing Greatly Varies Depending on Cell and Tissue Types**—We looked for possible quantitative and qualitative differences in the natural processing of PrPSc depending on which cells support the propagation of the prion. We first examined the molecular profile of the immunoreactive PrP species in different cell systems and mouse tissues infected by the same prion strain, 127S, a well characterized strain of sheep scrapie agent. The cell systems studied comprised previously described Rov and MovS cell lines, primary CGN and astrocytes (CAS) derived from tg338 mice (29), and the tissues were whole brain and spleen homogenates from tg338 mice. All these materials are genetically engineered to express solely the same ovine PrP\(^\text{RvQ}^\text{2}\) allotype. On immunoblot analysis, a typical profile, including a well individualized band of 26 kDa corresponding to unglycosylated PrP\(^C\), was observed in uninfected cell cultures and healthy tissues. In scrapie-infected Rov and MovS cells, additional PrP fragments shorter than 26 kDa were present, whose size matched those of PK-resistant PrPSc fragments (Fig. 1A). Centrifugation of cell lysates corroborated the presence of detergent-insoluble, truncated PrPSc, which was quantitatively recovered in the pellets, whereas normal PrP\(^C\) did not sediment in these conditions. As expected, sedimented PrPSc was also PK-resistant (data not shown). Therefore, the bulk of PrPSc accumulating in Rov and MovS cells consisted of naturally truncated molecules, shown below to be bona fide C2 (i.e. N-terminal-truncated) fragments. Upon quantification after deglycosylation by PNGase the full-length (FL) species were found to represent only 5 and 15% of PrPSc accumulated in Rov and MovS cells, respectively (see Fig. 12 for an overview of the quantification data). Rov cultures were maintained in serum-free medium and passed without using trypsin for splitting accumulated truncated PrPSc-like cultures kept in standard...
PrPSc Trimming Variation in Cell and Tissue Types

The image contains a figure with a legend for the figure, indicating it shows PrPSc trimming variation in cell and tissue types. The figure is labeled as "A" and "B" with various panels indicating the infection types and proteinase K conditions. The text provides details on the PrPSc processing and trimming in different cell and tissue types, discussing the variations and the effects of proteinase K digestion on PrPSc molecules.

The text mentions that PrPSc molecules are detected in infected cells and tissues, and the processing of PrPSc varies depending on the cell type. PrPSc molecules are detected in brain homogenates, and the processing is influenced by the presence of proteinase K treatment. The figure shows the electrophoretic mobility of PrPSc molecules under different conditions, indicating the presence of both full-length and truncated species.

The text further explains that PrPSc molecules are detected in infected cell lines and primary neurons, and the processing is influenced by the presence of proteinase K treatment. PrPSc molecules are also detected in the brain and spleen homogenates from infected mice, and the processing is influenced by the presence of proteinase K treatment.

Overall, the text and figure provide a comprehensive overview of PrPSc trimming variation in cell and tissue types, highlighting the differences in PrPSc processing under different conditions and the effects of proteinase K digestion on PrPSc molecules.
**FIGURE 2.** CGN accumulate thermolysin-resistant, full-length PrP\(^{Sc}\). Whole lysates from uninfected (lanes 1 and 2 of each panel) and 1275-infected (lanes 3–5) tg338 CGN, brain, and Rov cultures were digested with thermolysin (≥4 experiments) using conditions in which PrP\(^{C}\) was completely proteolyzed (lanes 2 and 4). Immunoblots using the Sha31 mAb show that thermolysin-resistant PrP\(^{Sc}\) from CGN cells and the brain migrates with the mobility of FL species (lanes 4), not with PrP\(^{Sc}\) generated by the PK treatment (lanes 5). In contrast, the profiles of thermolysin- and PK-resistant PrP\(^{Sc}\) from Rov cells are similar (compare lanes 4 and 5 in Rov panel). The positions of molecular mass markers are indicated.

**FIGURE 3.** Truncated PrP\(^{Sc}\) from MovS cells binds to the IMAC-Cu\(^{2+}\) column. Lysates of MovS cells (lane T) were loaded onto an IMAC-Cu\(^{2+}\) column. After recovery of unbound proteins (FT, flow-through), bound proteins were eluted by application of a 3–200 mM linear imidazole gradient and fractions (numbers 2–15) were analyzed by immunoblotting using either Sha31 mAb that recognizes the PrP core (A, B, and D) or anti-octarepeat Pc248 mAb (C). PrP elution profiles obtained with non-infected (A) and infected cells before (B and C) or after PK digestion of the eluted fractions (D) are shown. Pc248 mAb detects non-truncated PrP in infected cells (C), whereas Sha31 detects both truncated and FL molecules (B). PK digestion (D) proves that the population of truncated PrP corresponds to the bulk of PrP\(^{Sc}\) produced by MovS cells.

noblots were performed using ICSM4 antibody specific for unglycosylated PrP. These experiments confirmed the slower mobility of the PK-resistant fragments from CGN cells and brain tissue compared with Rov and MovS cells (Fig. 5A). A similarly slower migration of PrP\(^{Sc}\) fragments from the brain versus spleen tissue was also reproducibly observed (see Fig. 1B). These experiments also revealed a variation in the mobility of PrP\(^{Sc}\) in corresponding, non-infected samples. To determine which cell-specific, post-translational modification of PrP\(^{C}\) was involved, lysates from uninfected cultures or brain tissue were treated with hydrofluoric acid to remove the GPI moiety (Fig. 5B). Because after this treatment PrP\(^{C}\)-unglycosylated bands migrated uniformly, it was concluded that a variation in the composition of the GPI anchor is mainly responsible for the observed mobility differences of PrP\(^{C}\).

However, based on the relative mobility we calculated that the difference between PK-resistant fragments slightly exceeded that between PrP\(^{C}\) species (i.e. 2.1 versus 1.0 kDa, respectively, data not shown), thus suggesting that the observed differential mobility was not ascribable solely to PrP\(^{C}\). Relevant monoclonal antibodies were used to probe epitopes of PrP\(^{Sc}\) present in the different cell cultures and tissues after PK digestion, and typical results are presented in Fig. 6. Whereas antibodies 12B2 (position 93–97 in sheep sequence) and CT179 (position 218–231) both produced strong signals in all cases, clear differences were observed with antibody Pc248. This antibody, previously described by our laboratory (32, 44), binds PrP within the octarepeat region with a very high avidity (epitope mapping and comparison with other anti-octarepeat mAbs are shown in supplemental Fig. S2). PK-resistant fragments from Rov and MovS cells were not recognized by Pc248, contrary to those from primary neurons or brain tissues (Fig. 6B), thus providing clear evidence that an octarepeat motif, still present in CGN cells and brain PrP\(^{Sc}\) after PK digestion, was lost in these cell lines. PK-resistant fragments from CAS exhibited some reactivity to Pc248 (Fig. 6C). When immunoprophing was performed on sedimented, non-PK-digested materials (Fig. 6D), CAS cell lysates were shown to contain a mixture of Pc248-negative and -positive (FL) PrP\(^{Sc}\) molecules, whereas in spleen homogenates FL PrP\(^{Sc}\) (Pc248-positive) was hardly detectable. Rov and MovS sedimented, undigested material failed to react with Pc248, confirming that a nearly exhaustive trimming of the PrP\(^{Sc}\) molecules rather than PK digestion accounted for loss of the Pc248 epitope in these cells. Altogether, these results established that N-terminal trimming of PrP\(^{Sc}\) molecules generated by ovine prion produces PrP fragments distinguishable from those resulting from the exogenous PK cleavage of FL PrP\(^{Sc}\).

**Differential PrP\(^{Sc}\) Trimming Occurs in Mouse Prion-infected Cells and Tissues**—To see whether our findings would extend to other than ovine prions, we next focused our study on various models propagating mouse prions. The CAD cell line originating from brain tissue displays several features of neurons (28, 38). CAD cultures steadily infected by 139A, 22L, or Chandler prion strains were shown to produce naturally N-terminal-truncated, Pc248-negative PrP\(^{Sc}\), which was predominantly monoglycosylated as is typically the case for PK-resistant PrP\(^{Sc}\) for these strains (Fig. 7A). Additional species of lower mobility were observed in sedimented material using Sha31 antibody (Fig. 7A), suggesting accumulation of FL PrP\(^{Sc}\) too. Indeed, sedimented, non-PK treated samples contained Pc248-reactive, FL PrP\(^{Sc}\) material, with predominant monoglycosylated function.
forms, and Pc248-reactive species were resistant to thermolysin digestion (Fig. 7B). The full-length form represented around one-third of PrPSc in these cells (see Fig. 12). Infected cultures of Rom cells, similar to Rov but genetically engineered to express mouse instead of ovine PrP (26), were shown to accumulate PrPSc essentially under the C2, Pc248-negative form (supplemental Fig. 3), as did the Rov cells. In contrast, truncated species were hardly detectable in 139A- or 22L-infected, primary cultured CGN mouse neurons (Fig. 7C).

In tissues of 139A-infected tga20 mice, PrPSc was shown to accumulate as a FL species in a great majority in the brain (~80%), whereas it appeared to be extensively trimmed in the spleen (Figs. 8 and 12), i.e. a situation similar to that seen in tg338 mice. Altogether these results indicated that the phenomenon of differential trimming is not a unique feature of ovine prions.

Inhibition of Lysosomal Hydrolases but Not of Calpains Restores Accumulation of Full-length PrPSc—To test whether the endolysosomal compartment was involved in trimming of PrPSc in the cell systems studied here, we used NH4Cl, a compound that inhibits protease activity by raising the pH in this compartment, and/or leupeptin, a drug commonly used to impair lysosomal hydrolases. Treatment of infected Rov cultures modified the PrP immunoreactive profile, with a decrease of the C2 species and a concomitant increase of the FL PrP species, obvious for the unglycosylated band; overall, the accumulation of PrPSc did not decrease or decreased only slightly (Fig. 9A, left two panels). Accumulation of FL PrPSc in the lysosome-impaired culture was further substantiated by detection
of thermolysin-resistant, detergent-insoluble, and Pc248-reactive PrP species (Fig. 9A, right three panels). Under lysosome inhibition the proportion of FL PrPSc increased from 5 to 58% and from 15 to 78% in Rov and MovS cells, respectively (see Fig. 12). Intriguingly, in a recent study, PrPSc trimming was reported to be impaired following inhibition of calpains, cysteine proteases that are not associated with the endolysosomal compartment, whereas inhibition of lysosomal proteases was ineffective (13). However, in neither Rov nor MovS cell cultures did treatment with ≥50 μM calpain inhibitor III (MDL28170) (13) affect PrPSc N-terminal trimming; at the highest dose, the inhibitor decreased the accumulation of PrPSc, yet the C2/FL ratio, as measured on sedimented material, remained unchanged (Fig. 9B).

Importantly, PrPSc fragments generated by PK digestion of lysates from NH4Cl-treated cells were recognized by Pc248 antibody, unlike that in control, untreated cultures (Fig. 9C). Therefore, PK-resistant fragments produced by digestion of FL PrPSc generated in endolysosome-impaired cells contained an octarepeat motif.

Similar experiments were performed in mouse prion-infected cells. Following treatment with NH4Cl, the proportion of FL PrPSc in 22L-infected cultures increased in a dose-dependent manner, and a 10 mM treatment led to the accumulation of an exclusively FL species in 139A-infected cells (Fig. 10A). When 22L-infected cells were cultivated for one passage in the presence of NH4Cl plus leupeptin, truncated PrPSc disappeared almost completely (Figs. 10, B and D, and 12); such cultures accumulated as much PK-resistant PrP as untreated cultures, implying that FL and truncated PrPSc had a similar resistance to PK digestion (Fig. 10B). Remarkably, blockade of N-terminal trimming was compatible with serial propagation of prion infection because the level of PrPSc remained fairly stable under sustained treatment over at least 6 passages (Fig. 10C). Upon discontinuation of the treatment, production of truncated forms resumed to its original level within one passage (supplemental Fig. S4). In CAD cell cultures treated with cathepsin inhibitor III the accumulation of C2 fragments was diminished, but again without an increase of the FL versus C2 species ratio (Fig. 10D, left panel). In contrast, inhibition of cathepsin L or B led to an increase of the FL species at the expense of trimmed fragments (Fig. 10D, right panel). Altogether, these results led us to conclude that the N-terminal trimming of PrPSc in ovine and mouse prion-infected cells primarily involves proteases other than calpains.

To further substantiate the nature of the enzymes involved in generation of the C2 fragment, FL PrPSc obtained following NH4Cl plus leupeptin treatment of infected Rov cells was subjected to in vitro digestion by cathepsin B or L, under two different buffer conditions; one mimicking the slightly acidic endosomal compartment (pH 6) and the other the acidic lysosomal compartment (pH 5) (Fig. 11) according to Jordans et al. (41). In both cases cleaved PrPSc fragments were obtained that showed a typical profile but lacked Pc248 reactivity (Fig. 11), as in Rov cell cultures whose endolysosomal compartment was unimpaired (see above).

In the mouse prion cell systems, CAD (Fig. 10B) and Rom cells (supplemental Fig. S3), endogenously trimmed, and PK-cleaved PrPSc fragments exhibited the same mobility. Indeed, contrary to that seen in ovine prion-infected cells, PrPSc fragments produced by PK digestion of FL PrPSc from lysosom-
impaired cells showed no reactivity toward Pc248 antibody (Fig 10B). Similarly, PrP\textsuperscript{res} in brain tissues from mice infected by 22L or other mouse prions, which mostly accumulate FL PrP\textsuperscript{Sc}, lacked Pc248 reactivity (data not shown). From these results it appears that, even though the main PK cleavage sites of sheep and mouse PrP\textsuperscript{Sc} differed, endogenous proteolytic cleavage generated C2 fragments with similar N termini, despite different cell types and PrP sequences.

**DISCUSSION**

The present study was focused on the natural processing of PrP\textsuperscript{Sc}, leading to N-terminal-trimmed fragments commonly called C2. Several new findings emerged that will be discussed successively below.

Our study first revealed that the efficiency of trimming varies dramatically depending on which cell or tissue supported replication of the infecting prion (see Fig. 12). Thus, in cell systems infectible by sheep prion, the epithelial Rov and Schwann MovS cell lines, the bulk of PrP\textsuperscript{Sc} consisted of molecules that were N-terminal-truncated prior to PK digestion. In primary cultured cells, PrP\textsuperscript{Sc} produced by neurons was essentially full-length, whereas in astrocytes an important proportion was trimmed. A contrasted situation was also observed in vivo (tg338 mouse model), where intact PrP\textsuperscript{Sc} molecules largely predominated in the brain, although they were minimally represented in the spleen. The above mentioned materials all expressed the same ovine prnp allele and were infected by the same, biologically cloned sheep prion, therefore excluding any sequence or strain-dependent conformational effect. Examination of models propagating mouse-adapted prions led to similar conclusions, indicating that this may be a general phenomenon.

Although in CAD neuronal cells, the proportion of C2 over FL molecules averaged two-thirds whatever the infecting prion strain, limited trimming occurred in primary cultured neurons. In the brain and spleen tissues of tga20 mice the FL/C2 ratios were roughly inverse, as seen in ovine PrP mice. It is reasonable
to anticipate that such a markedly tissue-dependent, differential PrP<sup>Sc</sup> trimming also exists in species naturally infected by prions.

Conceivably the endogenous cleavage of PrP<sup>Sc</sup>, and more specifically the generation of C2 fragments, might differ quantitatively between different nerve cell categories within the brain. Accumulation of C2 fragments in the brain tissue has been reported to occur in rodent models other than mice (13, 14), as well as in naturally affected host sheep (9) and humans (6, 45). Moreover, several studies have offered evidence potentially linking the C2 accumulation level in the prion-affected mouse, sheep, and human brains or its neuroanatomical deposition (7, 9, 14, 45) to TSE strain variation. Our observation that PrP<sup>Sc</sup> trimming efficiency in primary cultured astrocytes and neurons markedly differed, in particular, supports the view that certain nerve cell subpopulations might be more prone to endogenous proteolysis than others. Thus, the ratio of C2/FL PrP<sup>Sc</sup> species in the brain tissue could also reflect the cellular tropism or “targeting properties” of a prion, rather than the solely conformational specificities of PrP<sup>Sc</sup>. In future studies, it would be interesting to determine whether generation of the C2 fragments occurs preferentially in specific cell types within the central nervous system, and whether or not “high C2-producing” strains share common biological features.

It is generally considered that the C2 fragments are the in vivo counterparts of the protease-resistant fragments produced by PK digestion in vitro (6, 11, 13). However, the findings presented here do not support this view. As revealed by antibody mapping, C2 fragments consistently failed to react with the potent anti-octarepeat antibody Pc248, whatever the infecting prion. To be underlined, all the agents studied here exhibited molecular features of type 1 human prions (4, 5), i.e. PK-resistant PrP fragments around 21 kDa (unglycosylated band), being typically strongly reactive toward the 12B2 antibody (33). The observed lack of Pc248 reactivity of fragments generated by PK cleavage of FL PrP<sup>Sc</sup> appears to be a specific feature of mouse prions. Indeed, in other prion-infected species, PK cleavage of “type 1-like” PrP<sup>Sc</sup> tends to preserve C-proximal octarepeat residues. This is the case for sheep prions, as shown in this study by the strong Pc248 reactivity of PK-resistant fragments generated from FL PrP<sup>Sc</sup> (e.g. in brain tissue or primary neurons (CGN)). Consistent with our findings, the Trp-84 residue, located upstream of the Pc248 epitope sequence delineated here, was recently identified by mass spectrometry analysis as one of the major PK cleavage sites in brains of natural or experimental scrapie-affected sheep (46), whereas residues within or downstream of the Pc248 epitope were identified as the main PK cleavage sites in scrapie-infected mice (11, 47, 48). Recently, PK-resistant PrP<sup>Sc</sup> accumulating in the brain of human patients infected by type 1 prions (5, 49) was reported to be strongly reactive toward two newly introduced anti-octarepeat antibodies (50). We surmised that these antibodies (Pom2 and Pom12) have properties very similar to those of Pc248, which is characterized by high avidity for brain PrPres, whereas commonly used anti-octarepeat antibodies generally exhibit weak reactivity (for more information on Pc248 antibody, see supplemental Fig. S2). PK-resistant fragments retaining an octarepeat motif are produced upon infection by the 21K type of hamster prions also (51).<sup>5</sup> In conclusion, the equivalence between C2 and PK-cleaved fragments commonly observed with mouse prions is far from the general rule. Endogenous

<sup>5</sup> M. Moudjou, unpublished data.
processing events that precede in vitro proteolytic digestion determine the molecular features of 
PrP\textsuperscript{res} to a variable extent. This notion might be worth accounting for while interpreting 
PrP\textsuperscript{Sc} molecular profiles in biopsies from brain or other tissues 
that might markedly differ in their trimming capability.

PrP\textsuperscript{res} fragments in mouse and sheep prion-infected cell lines 
typically have a higher mobility than their counterparts in the 
brain tissue of wild type or ovine transgenic mice, respectively 
(25, 27, 52, 53). Re-inoculation to mice of the cell culture-prop-
agated agent revealed no permanent change of its molecular 
profile and strain properties (26, 27, 52–55). The phenomenon 
has been proposed to simply reflect post-translational differ-
ences of PrP\textsuperscript{C} molecules when expressed either in culture or 
tissue (52, 53). Thus, unglycosylated PrP\textsuperscript{C} in mouse cell lines 
migrate faster than mouse brain PrP\textsuperscript{C}, putatively reflecting dif-
ferent compositions of the GPI moieties (52). New findings of 
the present study offer clarification of the cause of these obser-
vations. Our results established that, whereas in mouse prion-
infected cells the N termini of endogenously and PK-cleaved 
PrP\textsuperscript{Sc} are similar (i.e. both Pc248-negative and 12B2-positive), 
a more complex situation prevails in sheep prion-infected cells; 
indeed, two factors appeared to account for the molecular mass 
difference observed between brain and cell PrP\textsuperscript{Sc} fragments: (i) 
a differential mobility of PrP\textsuperscript{C} due to the variable composition 
of the GPI moiety, which was abolished after removal of the 
lysosomal inhibitor. Cell lysates were immunoblotted using Sha31 mAb. Molecular mass markers in kDa are shown. D, infected CAD cells were treated 
with various concentrations of calpain inhibitor III, NH\textsubscript{4}Cl plus leupeptin, cathepsin L inhibitor I (cath.L), or cathepsin B inhibitor I (cath.B), as indicated. Sedimented materials from cell lysates were analyzed by Western blotting with Sha31 mAb. These experiments have been repeated three times with consist-
tent results. n.i., non-infected.

FIGURE 11. In vitro cleavage of full-length PrP\textsuperscript{Sc} by cathepsin B and L 
remove the Pc248 epitope. PrP\textsuperscript{Sc} from a lysate of infected Rov cells treated 
with NH\textsubscript{4}Cl and leupeptin was purified by sedimentation, resuspended in two 
different cathepsin buffers (pH 6 or 5), and digested or not (control lanes) with 
the indicated proteases as described under “Experimental Procedures.” The 
same samples were revealed with either Sha31 or Pc248 mAbs.

PrP\textsuperscript{Sc} Trimming Variation in Cell and Tissue Types

| A | B |
|---|---|
| NH\textsubscript{4}Cl (mM) | Proteinase K |
| 0 | + |
| 5 | + |
| 10 | + |
| 20 | + |

FIGURE 10. Lysosome inhibition blocks PrP\textsuperscript{Sc} N-terminal trimming but not prion propagation in CAD cells. A, non-infected, 22L- and 139A-infected cells 
were either left untreated or treated with NH\textsubscript{4}Cl as indicated, and cell lysates were centrifuged. The pellets were analyzed for their PrP\textsuperscript{Sc} content by immuno-
blotting using Sha31 mAb. B, 22L-infected CAD cells cultivated in the absence or presence of 10 mM NH\textsubscript{4}Cl plus 10 \mu g/ml of leupeptin for two consecutive 
passages. Whole cell lysates and sedimented materials (right two panels) were PK-digested or not before immunoblotting using Sha31 or Pc248 mAb. C, 22L-infected CAD cells were cultivated in the absence or presence of 10 mM NH\textsubscript{4}Cl for 4 days and cells were passaged twice a week in the absence or presence 
of the lysosomal inhibitor. Cell lysates were immunoblotted using Sha31 mAb. Molecular mass markers in kDa are shown. D, infected CAD cells were treated 
with various concentrations of calpain inhibitor III, NH\textsubscript{4}Cl plus leupeptin, cathepsin L inhibitor I (cath.L), or cathepsin B inhibitor I (cath.B), as indicated. Sedimented materials from cell lysates were analyzed by Western blotting with Sha31 mAb. These experiments have been repeated three times with consist-
tent results. n.i., non-infected.
were treated with thermolysin and further centrifuged in the case of spleen material to concentrate PrPSc. PrPres was reproducible, and plainly ion-dependent (data not length or naturally trimmed PrPSc molecules in the absence of any treatment that would denature or cleave the PrPSc species. From a theoretical viewpoint, our observation suggests that determinants of the N-terminal region are accessible within native PrPSc multimers, because FL PrPSc and PrPSc exhibited close avidities for bound copper.

Studying the effect of lysosomotropic compounds and protease inhibitors on the generation of C2 fragments has led to somewhat controversial observations in the literature. In all three cell lines examined here (Rov, MovS, and CAD), the formation of C2 fragments was blocked efficiently by leupeptin treatment and NH4Cl-induced pH elevation, with little or no effect on short term PrPSc accumulation. To be noted, PrPSc in lysosome-impaired Rov and CAD cell cultures consisted of true FL species, not partially protected species with a shorter size truncation (17). Our results, in line with earlier observations in two other cell lines (12, 17), thus provided robust evidence for the involvement of the endolysosomal compartment in the N-terminal trimming of PrPSc. Furthermore, we showed that in vitro cleavage of full-length PrPSc by cathepsins B and L produced fragments that lacked the Pc248 octapeptide epitope, as observed for the endogenously produced C2 fragments. Conversely, cathepsin inhibitors were found to diminish the formation of C2 fragments in infected cell cultures. These data bring direct evidence for the involvement of these hydrolases into the generation of C2 PrPSc.

Another unexpected finding in this study was the retention of non-denatured PrPSc by the copper ion, because failure to bind to immobilized copper ion has been reported by others to be a distinctive trait of non-denatured PrPSc (56, 57). In our experimental conditions, the binding of both FL PrPSc and PrPres was reproducible, and plainly ion-dependent (data not shown). It was observed irrespective of whether cell lysate or brain homogenate was used as a starting material. A strain-specific effect is implausible, because efficient binding was observed with hamster PrPSc also, the prion agent used in one previous study (56). Although we still have no clear explanation to offer for these discrepant data, we suggest reconsidering copper ion binding as a potentially useful means to prepare full-length or naturally trimmed PrPSc molecules in the absence of any treatment that would denature or cleave the PrPSc species. From a theoretical viewpoint, our observation suggests that determinants of the N-terminal region are accessible within

\[ 10262 \]

\[ JOURNAL \ OF \ BIOLOGICAL \ CHEMISTRY \]

\[ VOLUME \ 285 \ • \ NUMBER \ 14 \ • \ APRIL \ 2, \ 2010 \]

\[ M. \ Moudjou, \ unpublished \ results. \]

\[ FIGURE \ 12. \ Proportion \ of \ untrimmed \ PrPSc \ in \ infected \ cells \ and \ tissues. \ A, \ Western \ blot \ analysis \ (Sha31 \ mAb) \ of \ PNGase-treated \ samples: \ cell \ lines \ (Rov, \ Mov, \ CAD), \ primary \ cultures \ of \ neurons \ (CGN) \ or \ astrocytes \ (CAS), \ and \ brain \ and \ spleen \ tissues. \ Each \ of \ the \ three \ left \ panels \ show \ three \ samples \ left \ untreated \ and \ one \ sample \ treated \ with \ lysosomal \ inhibitors \ (10 \ mM \ NH4Cl \ plus \ 10 \ lg/ml \ leupeptin); \ the \ four \ right \ panels \ each \ show \ two \ different \ samples. \ Sedimented \ material \ from \ lyses \ of \ cell \ lines \ and \ primary \ cultures \ were \ resuspended \ and \ treated \ with \ PNGase \ (similar \ results \ were \ obtained \ using \ samples \ that \ were \ digested \ with \ thermolysin \ before \ PNGase; \ not \ shown). \ Lysates \ from \ tg338 \ brain \ and \ spleen \ tissues \ were \ treated \ with \ thermolysin \ and \ further \ centrifuged \ in \ the \ case \ of \ spleen \ material \ to \ concentrate \ PrPSc. \ Arrows \ indicate \ deglycosylated \ full-length \ (FL) \ and \ N-terminal-truncated \ (C2) \ PrPSc \ species. \ B, \ the \ histogram \ shows \ the \ percentage \ of \ full-length \ species \ over \ total \ PrPSc \ as \ determined \ by \ quantification \ of \ samples \ such \ as \ seen \ in \ A. \ Average \ values \ and \ standard \ deviation \ were \ determined \ from \ four \ to \ seven \ different \ experiments. \]
ably involve some form of PrPSc. Could the trimming of PrPSc influence its neurotoxic potential? The N terminus of PrPSc appears to be important for anti-apoptosis and antioxidant functions (58, 59), but what about PrPSc? Transgenic mice that express a deleted form of PrP mimicking C2 fragments can be infected and develop a lethal neurological disease, but this does not preclude the existence in the truncated region of determinants that could modulate the cytotoxicity of PrPSc. Interestingly, the neuropathology in one such transgenic line was reported to be profoundly modified, i.e. no histopathological lesions could be detected in the brain or brainstem of terminally ill animals, and the cytopathic anomalies were essentially restricted to neurons of the cervical spinal cord (60). Altogether our ex vivo observations raise the question of whether trimming the FL PrPSc could modulate its neurotoxic potential and thus exert some protective effect in the target cells. Although immortalized cells fail to recapitulate the deleterious effects occurring in vivo, primary cultured neurons show a gradually compromised survival upon prion infection (29). It is intriguing that in the latter, the FL PrPSc form largely predominates over the C2 form, whereas the opposite is commonly observed in immortalized cells even of neural origin. In vivo, prion infection causes no overt cell death of astrocytes, which showed good trimming capacity in primary cultures, or of lymphoectic cells, where PrPSc could be extensively endoproteolyzed, considering our results with splenic tissue. The processing of PrPSc systems, including blockade of PrPSc trimming without impairing prion replication, should make it feasible to further explore these aspects.

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Endogenous Proteolytic Cleavage of Disease-associated Prion Protein to Produce C2 Fragments Is Strongly Cell- and Tissue-dependent

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