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Uptake and Degradation of Protease-Sensitive and -Resistant Forms of Abnormal Human Prion Protein Aggregates by Human Astrocytes

Young Pyo Choi,* Mark W. Head,† James W. Ironside,† and Suzette A. Priola*

From the Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana; and the National Creutzfeldt Jakob Disease Research & Surveillance Unit,† School of Clinical Sciences, University of Edinburgh, Edinburgh, United Kingdom*

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Address correspondence to Suzette A. Priola, Ph.D., Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, 903 S. 4th St., Hamilton, MT 59840. E-mail: spriola@niaid.nih.gov.

Sporadic Creutzfeldt-Jakob disease is the most common of the human prion diseases, a group of rare, transmissible, and fatal neurologic diseases associated with the accumulation of an abnormal form (PrP^{Sc}) of the host prion protein. In sporadic Creutzfeldt-Jakob disease, disease-associated PrP^{Sc} is present not only as an aggregated, protease-resistant form but also as an aggregated protease-sensitive form ($sPrP^{Sc}$). Although evidence suggests that $sPrP^{Sc}$ may play a role in prion pathogenesis, little is known about how it interacts with cells during prion infection. Here, we show that protease-sensitive abnormal PrP aggregates derived from patients with sporadic Creutzfeldt-Jakob disease are taken up and degraded by immortalized human astrocytes similarly to abnormal PrP aggregates that are resistant to proteases. Our data suggest that relative proteinase K resistance does not significantly influence the astrocyte's ability to degrade PrP^{Sc}. Furthermore, the cell does not appear to distinguish between $sPrP^{Sc}$ and protease-resistant PrP^{Sc}, suggesting that $sPrP^{Sc}$ could contribute to prion infection. *(Am J Pathol 2014, 184: 3299–3307; http://dx.doi.org/10.1016/j.ajpath.2014.08.005)*

Prion diseases, or transmissible spongiform encephalopathies, are rare fatal neurologic disorders of mammals that include Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep. Prion diseases are characterized by the conversion of normal prion protein (PrP^{C}) into a disease-associated and aggregated isoform (PrP^{Sc}), which is thought to be the main component of the infectious agent or prion (reviewed in Priola and Vorberg[1]). PrP^{C} is a glycoprotein that contains two N-linked glycosylation sites[2,3] and is bound to the plasma membrane via a glycosyl-phosphatidyl-inositol anchor.[4] Although PrP^{C} is detergent soluble and fully susceptible to proteolytic degradation, PrP^{Sc} has an increased detergent insolubility and partial resistance to proteinase K (PK).[5] The presence of amino-terminally truncated, PK-resistant core fragments of PrP^{Sc} ($rPrP^{Sc}$) after limited proteolysis is considered the most reliable diagnostic marker for prion infection,[6,7] and biochemical profiles of $rPrP^{Sc}$ based on molecular mass and/or the degree of glycosylation are used to help differentiate distinct prion disease phenotypes in humans.[8–11]

In recent years, alternative approaches for analyzing PrP^{Sc} that do not rely on the enzymatic removal of PrP^{C} have indicated that not all forms of PrP^{Sc} are necessarily resistant to proteolytic treatment. The conformation-dependent immunoassay, which uses conformational differences between the N termini of PrP^{C} and PrP^{Sc}, has found that often a majority of PrP^{Sc} present in prion-affected brains is susceptible to proteolytic degradation.[12–15] In sporadic CJD (sCJD), this PK-sensitive species of aggregated PrP^{Sc}, termed $sPrP^{Sc}$, was found in some cases to account for up to 90% of the total PrP^{Sc}.[13,16] Careful analysis of the size distribution of PrP^{Sc} has also found that $sPrP^{Sc}$ forms much smaller aggregates than $rPrP^{Sc}$.[17,18] Thus, $sPrP^{Sc}$ appears to represent a population of PrP^{Sc} aggregates which tends to be both smaller and more protease sensitive than $rPrP^{Sc}$.

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Current address of Y.P.C., Korea Brain Research Institute, Daegu, Republic of Korea.

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Evidence suggests that, like $rPrP^{Sc}$, $sPrP^{Sc}$ has seeding activity and can convert PrP^{C} to protease resistance.[18,19] It has also been associated with prion infectivity[20] and may influence the incubation time of prion disease.[21] Although these data suggest that PK-sensitive disease-associated PrP aggregates may be actively involved in prion pathogenesis, no studies have been performed to determine how this population of PrP aggregates might interact with cells and influence prion infection. In this study, we have looked at the uptake and degradation of PK-sensitive and PK-resistant disease-associated PrP aggregates in an established human astrocyte cell line. Our results indicate that, despite their biochemical differences, PK-sensitive PrP aggregates are taken up and degraded similarly to PK-resistant PrP aggregates, suggesting that relative PK resistance does not significantly influence the cell's ability to degrade PrP^{Sc}. Thus, the astrocyte does not appear to distinguish between $sPrP^{Sc}$ and $rPrP^{Sc}$ aggregates, suggesting that $sPrP^{Sc}$ could be involved in prion pathogenesis.

Materials and Methods

Human Brain Material

Ethical approval for the acquisition and use of human brain material was obtained from the NIH Office of Human Subject Research (Exempt 5480). Human brain tissue derived from four patients with sCJD and two control patients with other non-CJD neurologic disorders were obtained from the National CJD Surveillance Unit Brain and Tissue Bank in Edinburgh, Scotland. All cases had consent for research, and their supply and use in this study was covered by LREC 2000/4/157 (National Creutzfeldt-Jakob disease tissue bank: acquisition and use of autopsy material for research on human transmissible spongiform encephalopathies, Professor James Ironside, amended October 9, 2007). The four sCJD cases used had been fully characterized and comprised two cases of the MM1 subtype and one each of the MV1 and the VV2 subtypes ($CJD1^{MM1}$, $CJD2^{MM1}$, $CJD3^{MV1}$, and $CJD4^{VV2}$, respectively) according to the nomenclature used by Parchi et al.[10] In each case, approximately 2 g of brain tissue taken from the frontal cortex was homogenized in phosphate-buffered saline (PBS; pH 7.4) to a final 10% weight/volume (w/v) homogenate by using a MiniBeadbeater-8 (BioSpec, Bartlesville, OK). The homogenate was divided into aliquots and stored at −80°C until use.

Cells

The established human astrocyte cell line SVG p12 (CRL-8621) was obtained from ATCC (Manassas, VA). SVG p12 cells (hereafter referred to as SVG cells) were maintained in Eagle's Minimal Essential Medium (EMEM; ATCC), supplemented with 10% fetal bovine serum (FBS; Gibco-Life Technologies, Grand Island, NY), 100 U of penicillin, and 100 μg of streptomycin (Gibco-Life Technologies) in a humidified chamber at 37°C with 5% CO_2. On receipt, the SVG p12 cells were passaged four times in medium in a humidified chamber at 37°C with 5% CO_2. At passage 16, a large batch of cells was frozen in 90% FBS/10% dimethyl sulfoxide and stored in liquid nitrogen until use. For each experiment, a vial of cells at passage 16 was thawed and passaged once, and the cells were plated at a density of 2×10^6 cells per well of a six-well plate and allowed to attach overnight. After removal of medium, cells were overlaid with 800 μL of 1:10 dilution (in EMEM) of 10% brain homogenate. After 4 hours, 2 mL of EMEM plus 10% FBS was added. At 2, 8, 24, or 48 hours after exposure, cells were washed thoroughly with fresh medium and directly lyzed with 500 to 550 μL of 2% sarkosyl/PBS. In experiments in which the degradation of PrP^{Sc} was assayed, the initial inoculum was removed at 2 hours after infection, and the cells were washed extensively. After the addition of 3 mL of EMEM plus 10% FBS, cells were incubated for up to 24 hours and then lyzed.

For immunofluorescence studies, 3×10^4 SVG cells were plated into each well of a Lab-Tek Permanox 8-well chambered slide (Thermo Scientific, Rockford, IL) and allowed to attach overnight. After removal of the medium, the cells were overlaid with 125 μL of a 10% brain homogenate from the $CJD1^{MM1}$, $CJD2^{MM1}$, or non-CJD sample diluted 1:10 in Optimem (Gibco-Life Technologies). As an untreated control, cells were incubated in 125 μL of Optimem alone. After 4 hours, 400 μL of EMEM plus 10% FBS was added, and the cells were incubated an additional 20 hours.

NaPTA Precipitation

Sodium phosphotungstic acid (NaPTA; Sigma-Aldrich, St. Louis, MO) precipitation was performed as described previously[22] with minor modifications. Briefly, for SVG cell samples 500 μL of cell lysate was mixed with an equal volume of 2% sarkosyl/PBS. For brain samples, 50 μL of 10% homogenate was mixed with an equal volume of 4% sarkosyl/PBS, and the volume was brought to 1 mL by adding 900 μL of 2% sarkosyl/PBS. In some experiments, the relative amount of cell-associated PrP aggregates was estimated by comparison with an SVG cell lysate spiked with brain homogenate equivalent to 10% of the input inoculum. All samples were treated with 50 U/mL benzonase (Sigma-Aldrich), and NaPTA was added to a final concentration of 0.3% (w/v). The samples were then incubated at 37°C for 1 to 2 hours, followed by 30 minutes of centrifugation at 16,100 × *g*. Pellets were resuspended in 0.1% sarkosyl/PBS and, when appropriate, digested with 50 μg/mL PK for 30 minutes at 37°C. In some experiments, PK digestion was performed before the NaPTA precipitation.

Electrophoresis and Western Blot Analysis

Samples were boiled in urea sample buffer (62.5 mmol/L Tris, pH 6.8, 3 mmol/L EDTA, 5% glycerol, 5% SDS, 0.02% bromophenol blue, and 4 mol/L urea) for 10 minutes.

Samples were electrophoresed in Novex 10% Bis-Tris NuPAGE gels (Life Technologies, Carlsbad, CA) and then blotted on polyvinylidenedifluoride Immobilon-P membranes (Millipore, Rochester, NY). Subsequently, the membrane was blocked with 5% (w/v) dry milk in TBST (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween-20). PrP was detected by using either a 1:10,000 dilution of the anti-PrP mouse monoclonal antibody 3F4 conjugated to biotin (Covance Research Products, Denver, PA), followed by incubation in streptavidin-conjugated horseradish peroxidase (Cell Signaling Technology, Beverly, MA) at a dilution of 1:250,00 or a 1:1000 dilution of the monoclonal antibody 3F4 derived from a hybridoma cell supernatant made in house, followed by incubation in horseradish peroxidase–conjugated anti-mouse IgG F(ab') (GE Healthcare Life Sciences, Buckinghamshire, UK) at a 1:40,000 to 1:200,000 dilution. Both primary and secondary antibodies were diluted either in TBST or 1% (w/v) dry milk in TBST. Blots were developed by using ECL Plus, ECL Prime (GE Healthcare Life Sciences), or the SuperSignal West Femto Substrate (Thermo Scientific). The amount of protein on the blot was quantified by using ImageQuant software version 5.2 (Molecular Dynamics, Sunnyvale, CA). The percentage of PK-resistant PrP aggregates in each sample was calculated by dividing the amount of protease-resistant $rPrP^{Sc}$ in the NaPTA pellet by the total amount of PrP in the NaPTA pellet and then multiplying by 100. The percentage of PK-sensitive PrP aggregates in the sample was obtained by subtracting the percentage of $rPrP^{Sc}$ from 100.

For Western blot analysis of PrP^{C}, a confluent 25-cm^2 flask of either SVG cells or mouse fibroblast cells that expressed mouse PrP^{C} with the epitope to the mouse monoclonal antibody 3F4 (Mo3F4-ψ2C4[23]) were lyzed in 1 mL of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mmol/L Tris-HCL, pH 7.4, 150 mmol/L sodium chloride, 5 mmol/L EDTA). The lysate was centrifuged at 14,500 rpm for 5 minutes, and the supernatant was removed for further analysis.

Radiolabeling and Immunoprecipitation of PrP^{C}

SVG and Mo3F4-ψ2C4 cells were labeled with ^{35}S-methionine/cysteine, according to previously published techniques.[24] Briefly, confluent cells in a 25-cm^2 tissue culture flask (Corning, Corning, NY) were preincubated in methionine/cysteine-deficient RPMI (ICN Biomedicals, Aurora, OH) that contained 5% FBS and 4 mmol/L L-glutamine (Life Technologies) with or without 20 μg/mL tunicamycin (Calbiochem, Darmstadt, Germany). After 1 hour, 150 μCi of ^{35}S-methionine/cysteine (PerkinElmer, Waltham, MA) was added, and the cells were incubated an additional 2 hours. For cells treated with phosphatidylinositol phospholipase C (PIPLC), the radiolabeling time was 90 minutes, followed by a 30-minute chase in complete medium (RPMI + 10% FBS, 2 mmol/L L-glutamine, 100 U of penicillin, 100 μg of streptomycin). After the chase, cells were rinsed and incubated a further 30 minutes in 1 mL of phosphate-buffered balanced salt solution plus 1.8 U/mL PIPLC (MP Biomedicals, LLC, Santa Ana, CA). For all samples, PrP^{C} was immunoprecipitated from either cell lysate or cell supernatant as previously described.[24] After immunoprecipitation, some samples were also treated with the deglycosylating enzyme PNGaseF (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Samples were run on Novex 16% Tris-Glycine SDS-PAGE gels (Life Technologies), and the gels were fixed in 50% methanol/10% acetic acid, followed by drying at 70°C under vacuum. The dried gels were developed with Molecular Dynamics low-energy Phosphor Imager screens and quantified with ImageQuant software version 5.2 (Molecular Dynamics).

Immunofluoresence

Cells were stained for PrP^{Sc} as described previously[25] except that, because of the low level of PrP^{C} expression in SVG cells, digestion of the cells with PK was omitted. After treatment with 4 mol/L guanidine thiocyanate for 30 minutes at room temperature, PrP^{Sc} was detected by using a 1:50 dilution of the mouse monoclonal antibody 3F4 conjugated to biotin, followed by development with a 1:400 dilution of streptavidin conjugated to Alexa Flour 594 (Life Technologies). LAMP2b was directly stained by using a 1:50 dilution of a rabbit polyclonal anti-LAMP2b antibody conjugated to Alexa Fluor 488 (Novus Biologicals, Littleton, CO). Slides were analyzed with a Nikon Eclipse 55 microscope with a Nikon Internalight C-HGF1 fluorescence source (Nikon, Mellville, NJ). Images were taken with a Nikon Digital Sight camera and Nikon NIS-Elements Imaging Software AR version 3.2 by using the same camera settings with autoscaling enabled.

Results

Relative Amount of Protease-Resistant and Protease-Sensitive PrP^{Sc} in sCJD Brain Samples

We first examined the sCJD samples to determine the relative amounts of PK-sensitive and PK-resistant aggregated PrP recovered by NaPTA precipitation. PrP aggregates precipitated by NaPTA were left undigested or digested with PK and then analyzed by immunoblot (Figure 1A). More than 90% of the PrP aggregates in the $CJD1^{MM1}$ sample were PK resistant (Figure 1B). In sharp contrast, most PrP aggregates in the samples from the other three CJD cases were sensitive to PK digestion with only 20% to 25% of the PrP aggregates resistant to PK treatment (Figure 1B). Because only a small population of PrP present in uninfected brains was precipitable by NaPTA[26] (Figure 1A), protease-sensitive PrP aggregates in sCJD samples that were recovered by NaPTA were considered to largely represent $sPrP^{Sc}$.[13] Our data were consistent with other studies in which most PrP^{Sc} in sCJD brains was often $sPrP^{Sc}$.[13,16,21]

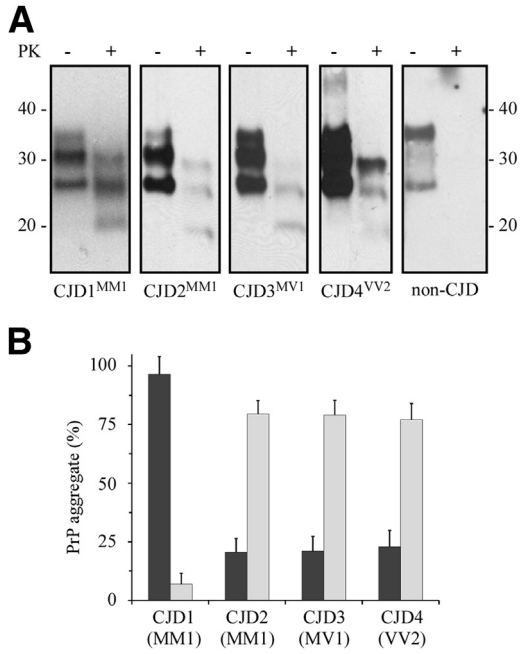

Figure 1 PK-resistant and -sensitive fractions of PrP aggregates in sCJD brains. **A:** PrP aggregates were recovered from sCJD-affected or non-CJD brains by using NaPTA precipitation. PrP aggregates were left undigested (−) or were digested (+) with PK and were analyzed by Western blot analysis using the anti-PrP monoclonal antibody 3F4. **B:** Quantitation of the relative amounts of PK-sensitive (light gray bar) and PK-resistant (dark gray bar) PrP aggregates in sCJD brain. Data are expressed as means ± SEM. $n = 2$ (**B**, $CJD2^{MM1}$ and $CJD3^{MV1}$) or $n = 3$ (**B**, $CJD1^{MM1}$ and $CJD4^{VV2}$) experiments.

Expression of PrP^C in SVG Cells

The human astrocyte cell line SVG was used to study the uptake of human PrP^{Sc}. Because formation of new PrP^{Sc} (ie, acute formation) in SVG cells exposed to sCJD brain material could confound the analysis of the uptake of the input PrP^{Sc}, we compared PrP^C expression in SVG cells with that in Mo3F4-ψ2C4 cells, a cell line known to support acute PrP^{Sc} formation.[23] Analysis by immunoblot found that PrP^C was present at detectable but low levels compared with PrP^C expressed in Mo3F4-ψ2C4 cells (Figure 2A). Consistent with the immunoblot results, ^{35}S-labeled PrP^C could also be immunoprecipitated from SVG cells as indicated not only by the presence of a band that comigrated with unglycosylated PrP^C in Mo3F4-ψ2C4 cells (Figure 2B) but also by the presence of fully glycosylated PrP^C (Figure 2B). However, quantitation of radiolabeled PrP^C found that its expression level in SVG cells was extremely low, representing only 3% of that in Mo3F4-ψ2C4 cells. Furthermore, in SVG cells only 6% of PrP^C was PIPLC releaseable (Figure 2B). This is the pool of PrP^C which is required for the formation of PrP^{Sc} in cells.[24] By contrast, in Mo3F4-ψ2C4 cells that can acutely make PrP^{Sc},[23] 70% of the PrP^C expressed was PIPLC releasable (Figure 2B). The low level of overall PrP^C expression in SVG cells, coupled with the extremely low levels of PIPLC-releasable PrP^C on the cell surface, indicated that acute formation of PrP^{Sc} would be either undetectable or minimal and thus would be unlikely to significantly affect our analysis of PrP^{Sc} uptake.

Aggregated PrP in Uninfected Brains Does Not Associate with SVG Astrocyte Cells

The small population of PrP present in uninfected brains that was precipitated by NaPTA (Figure 1A) could confound any analysis of cell-associated sCJD PrP^{Sc}. To determine whether PrP recovered from SVG astrocyte cells by NaPTA precipitation was specifically associated with prion infection, we first exposed cells to non-CJD brain homogenate. At different time points after exposure cells were lyzed, the lysates were precipitated with NaPTA, digested with PK or left undigested, and then analyzed by immunoblot. PrP aggregates were not detectable at any time point assayed (Figure 3A). Thus, the PrP aggregates present in the non-CJD brain homogenate either did not associate with astrocytes or the amount associated with the cells was below the level of detection of our immunoblot assay.

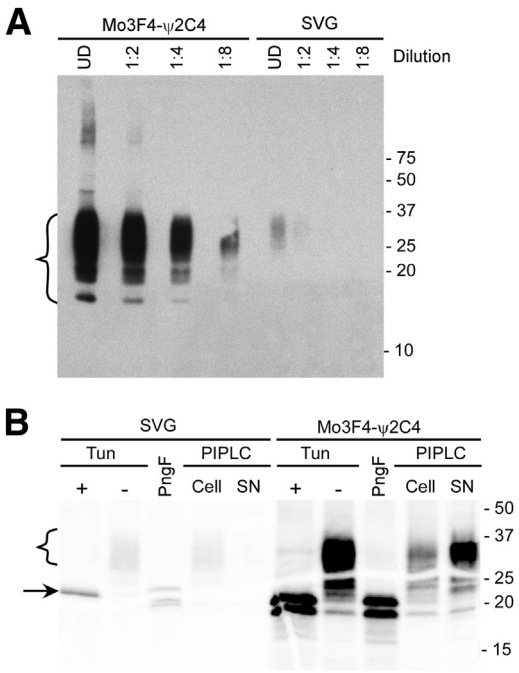

Figure 2 Low level of PrP^C expression in human astrocyte SVG cells. SVG cell line and mouse fibroblasts expressing mouse PrP^C that contained the epitope to the monoclonal antibody 3F4 (Mo3F4-ψ2C4) were assayed for PrP^C by either immunoblot analysis using the anti-PrP mouse monoclonal antibody 3F4 conjugated to biotin (**A**) or by radiolabeling using ^{35}S-methionine/cysteine in the presence (+) or absence (−) of Tun or PIPLC (**B**). Following radiolabeling, PrP^C was immunoprecipitated by the anti-PrP mouse monoclonal antibody 3F4. After immunoprecipitation, some samples were also treated with the deglycosylating enzyme PngF. The **bracket** indicates glycosylated PrP^C, and the **arrow** indicates unglycosylated PrP^C. The doublet in Mo3F4-ψ2C4 cells treated with either Tun or PngF likely represents both full-length and N-terminally truncated PrP^C. The lack of a doublet in Tun-treated SVG cells suggests that little or no PrP^C is truncated in these cells. Molecular mass markers are indicated on the right. Dilution, serial twofold dilutions of cell lysate; PngF, PNGaseF; Tun, tunicamycin; UD, undiluted.

Protease-Resistant and Protease-Sensitive PrP^{Sc} from Multiple sCJD Types Associate with SVG Astrocyte Cells

To examine whether the PK-sensitive PrP aggregates associated with sCJD interact with cells similarly to PK-resistant PrP aggregates, SVG astrocyte cells were incubated with brain homogenates prepared from sCJD brains. Cells were incubated with brain homogenate prepared from CJD1^{MM1} for up to 48 hours, and PrP aggregates associated with the cell were analyzed by Western blot analysis after NaPTA precipitation. Approximately 60% of the NaPTA-precipitated PrP aggregates associated with the cells 2 hours after exposure to CJD1^{MM1} were protease resistant, whereas approximately 40% were protease sensitive (Figure 3B). However, as the amount of cell-associated PrP aggregates increased over time, >90% of the PrP aggregates from 8 to 48 hours were resistant to PK (Figure 3B). Thus, with the exception of the 2-hour time point, the amount of cell-associated CJD1^{MM1} PrP aggregates was similar before and after PK digestion and reflected the predominance of PK-resistant PrP aggregates in the starting sample.

We next exposed SVG cells to brain homogenates prepared from the CJD cases CJD2^{MM1}, CJD3^{MV1}, or CJD4^{VV2} in which most PrP aggregates precipitated by NaPTA were susceptible to proteolysis. For all three sCJD cases, PrP aggregates again associated with the cell as early as 2 hours after exposure (Figure 3, C–E) with approximately 10% to 20% of the total input PrP becoming cell associated between 8 and 48 hours (Figure 3, C–E). Unlike CJD1^{MM1}, however, the amount of cell-associated PrP aggregate was greatly reduced after proteolysis. For the cells exposed to CJD3^{MV1}, the relative amount of PK-resistant PrP aggregates was similar at all time points tested and represented approximately 20% to 25% of all cell-associated PrP (Figure 3D). Similarly, the amount of PK-sensitive PrP aggregates associated with the cells remained steady at 75% to 80% (Figure 3D).

In SVG cells exposed to CJD2^{MM1} or CJD4^{VV2}, PK-sensitive PrP aggregates clearly associated with the cells at all time points tested (Figure 3, C and E). PK-resistant PrP aggregates were also detectable, but only after longer exposure of the blot (Figure 3, C and E). Thus, quantitation of the relative amounts of PrP before and after proteolysis was not possible. However, although there did appear to be an increase in PK-resistant PrP aggregates associated with the cell from 2 to 24 hours, the predominance of PK-sensitive PrP aggregates was consistent with the higher percentage of this form of PrP aggregate in the starting brain homogenates for both CJD2^{MM1} and CJD4^{VV2} (Figure 1B). Collectively, for all four sCJD cases examined in this study, the relative distribution of PK-sensitive and -resistant forms of PrP aggregates was the same whether it was present in the brain or associated with the cells, indicating that both forms have similar tendencies to become associated with SVG astrocyte cells.

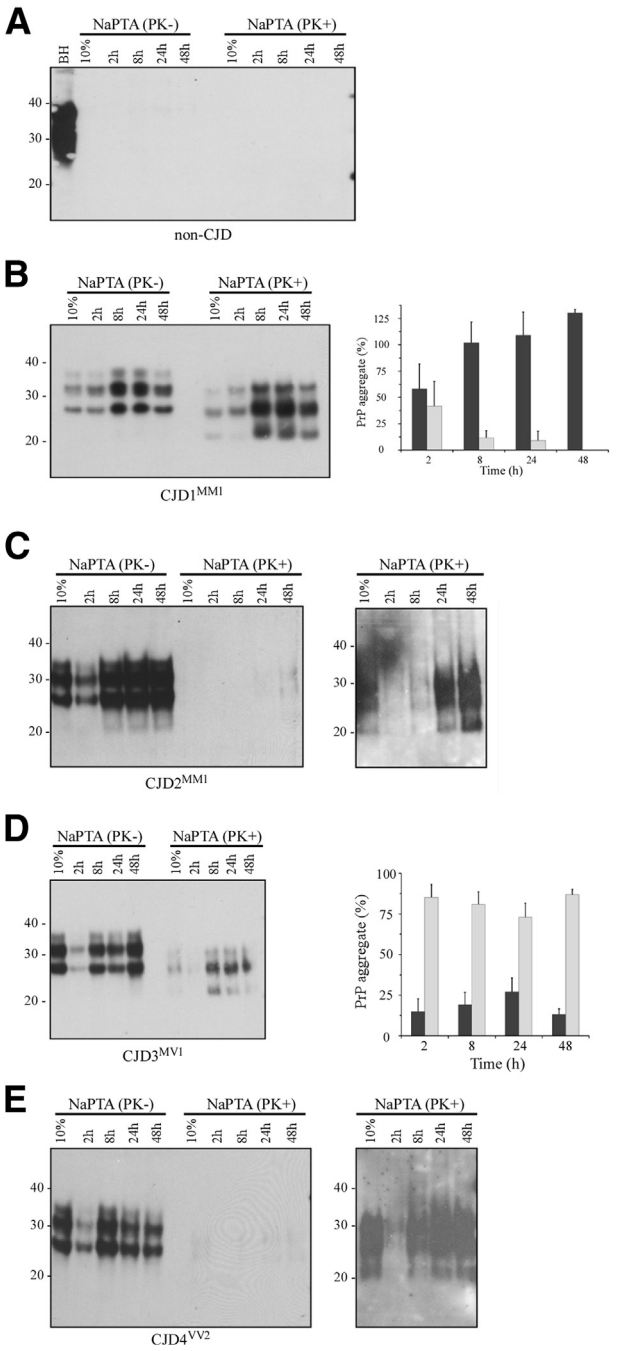

Figure 3 Both PK-resistant and -sensitive forms of CJD-specific PrP aggregates are associated with human astrocyte SVG cells. SVG cells were exposed to non-CJD (**A**), CJD1^{MM1} (**B**), CJD2^{MM1} (**C**), CJD3^{MV1} (**D**), or CJD4^{VV2} (**E**) brain homogenates for the times indicated. Cell-associated PrP aggregates were recovered as described in *Materials and Methods* and were analyzed by Western blot analysis with the use of the anti-PrP monoclonal antibody 3F4. For comparison purposes, lanes labeled 10% were loaded with a NaPTA precipitate from an SVG cell lysate spiked with brain homogenate equivalent to 10% of the input inoculum. **B** and **D:** Graphs show the relative amounts of PK-sensitive and PK-resistant PrP aggregates. Light gray bars, PK-sensitive PrP; dark gray bars, PK-resistant PrP. **C** and **E:** A longer exposure of the portion of the blot containing the PK-digested samples is shown on the right. For each immunoblot panel, molecular mass markers are shown on the left. Data are expressed as means ± SEM (**B** and **D**). $n = 3$ (**B**); $n = 5$ (**D**). BH, non-CJD brain homogenate equivalent to 10% of the input inoculum without any NaPTA precipitation.

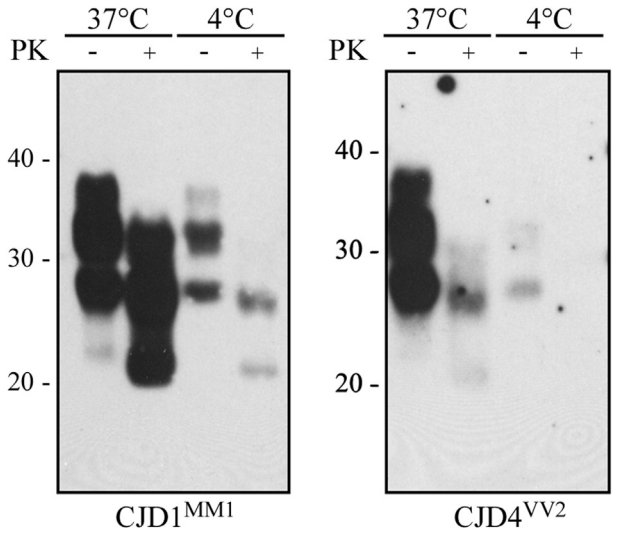

Figure 4 PK-resistant and -sensitive forms of disease-associated PrP aggregates are internalized by human astrocyte SVG cells. SVG cells were incubated with $CJD1^{MM1}$ or $CJD4^{VV2}$ brain homogenates at either 37°C or 4°C for 4 hours. Cells were lyzed, and the lysates were precipitated with NaPTA and digested with PK (+) as indicated. The amount of PrP aggregates internalized by the cell was determined by Western blot analysis with the use of the anti-PrP monoclonal antibody 3F4. Molecular mass markers are shown on the left.

PrP^{Sc} Is Actively Taken Up by SVG Astrocyte Cells

Next, we asked whether the PK-sensitive form of aggregated PrP associated with the SVG cell was being actively internalized. SVG cells were exposed to $CJD1^{MM1}$ or $CJD4^{VV2}$ brain homogenates and incubated either at 4°C, a temperature known to block active endocytosis of PrP^{Sc},[25,27] or 37°C. For cells exposed to $CJD1^{MM1}$ in which PrP aggregates consisted mostly of the PK-resistant form, the amount of PrP aggregates was greatly reduced in the cells incubated at 4°C compared with the cells incubated at 37°C (Figure 4). This result suggested that PK-resistant PrP aggregates were being taken up by cells via active endocytosis. For the cells exposed to $CJD4^{VV2}$, in which most PrP aggregates were sensitive to PK digestion, the amount of PrP aggregate associated with the cell at 4°C was, again, greatly reduced compared with the cells incubated at 37°C (Figure 4).

Immunohistochemistry was used to confirm that PrP^{Sc} was being internalized by the cells. SVG cells exposed to non-CJD, $CJD1^{MM1}$, or $CJD2^{MM1}$ brain homogenate for 24 hours were costained by using the anti-PrP mouse monoclonal antibody 3F4 and an antibody to LAMP2b, a marker for late endosomes/lysosomes. Previous work has reported that human PrP^{Sc} taken up by cells colocalizes with LAMP2b.[28] Consistent with the low level of expression of PrP^{C} in SVG cells, no PrP was detected in SVG cells incubated in medium alone (Figure 5). By contrast, and consistent with the internalization of PrP^{Sc}, there was minimal colocalization of PrP^{C} with LAMP2b in cells exposed to non-CJD brain homogenate but significant colocalization with LAMP2b in cells exposed to either $CJD1^{MM1}$ or $CJD2^{MM1}$ (Figure 5). Overall, our results strongly suggested that PrP^{Sc} was being actively internalized by SVG astrocyte cells.

Both Protease-Resistant and Protease-Sensitive PrP^{Sc} Are Degraded by SVG Astrocyte Cells

The observation that the relative ratio of cell-associated protease-sensitive to protease-resistant PrP aggregates remained largely unchanged during 48 hours (Figure 3D) suggested that the protease-sensitive PrP aggregates were not being degraded more rapidly than the protease-resistant PrP aggregates. To determine whether PrP aggregates susceptible to proteolysis were degraded similarly to those resistant to proteolysis, the $CJD3^{MV1}$ brain homogenate, in which most PrP aggregates were sensitive to PK digestion, was overlaid onto SVG cells. After a 2-hour incubation at 37°C, the cells were extensively washed to remove any remaining brain homogenate (0 hour) and incubated for an additional 6 and 24 hours in medium without any brain homogenate. Most PK-resistant PrP aggregates were associated with the cells after 6 hours of incubation but, by 24 hours, the amount of PK-resistant PrP had decreased by approximately 80% (Figure 6, A and B). Interestingly, the clearance pattern of the total PrP aggregates was similar to that of the PK-resistant aggregates with approximately 20% of the total PrP signal remaining after 24 hours (Figure 6B).

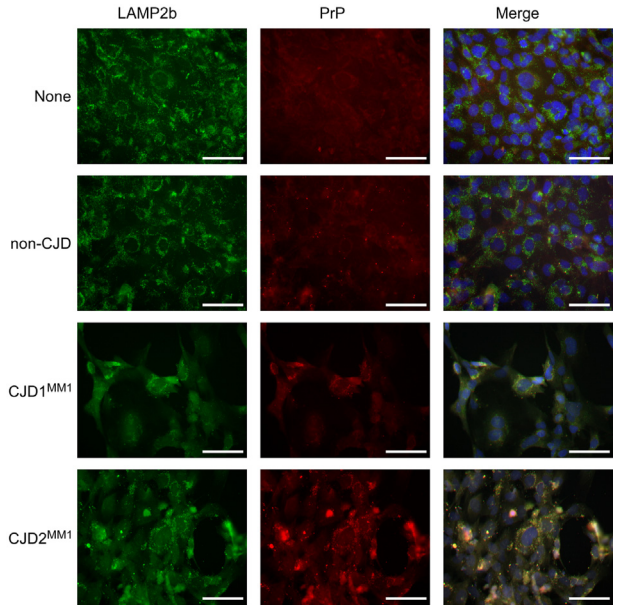

Figure 5 PrP^{Sc} taken up by human astrocyte SVG cells colocalizes with the endosomal/lysosomal marker LAMP2b. SVG cells were incubated in media alone (None) or non-CJD, $CJD1^{MM1}$, or $CJD2^{MM1}$ 1% brain homogenates. After 24 hours, all cells were treated with 4 mol/L guanidine thiocyanate to enhance the immunoreactivity of PrP^{Sc}. Cells were then costained for PrP (red) by using the anti-PrP mouse monoclonal antibody 3F4 conjugated to biotin, followed by streptavidin conjugated to Alexa Fluor 594, and for LAMP2b (green) by using an anti-LAMP2b rabbit polyclonal antibody directly conjugated to Alexa Fluor 488. Scale bar = 50 μm. Original magnification, ×400.

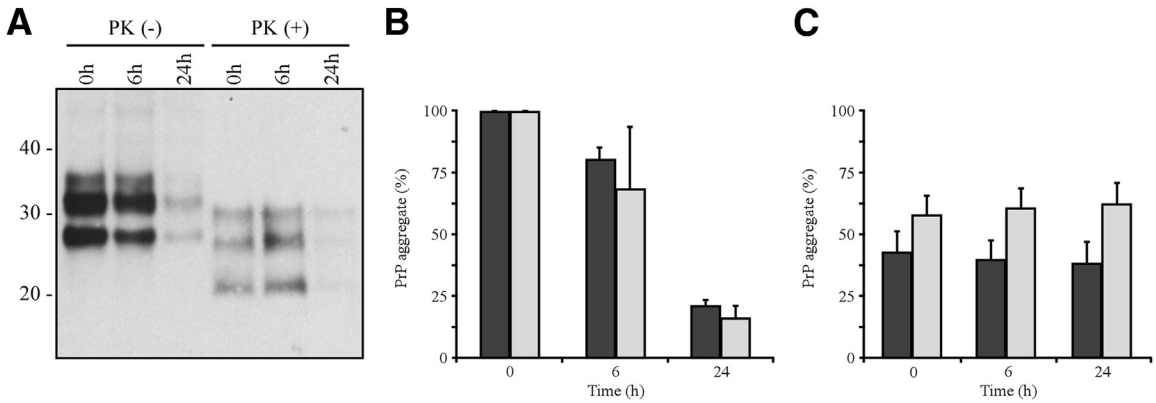

Figure 6 PK-resistant and -sensitive forms of disease-associated PrP aggregates are degraded by cells at a similar rate. Human astrocyte SVG cells were incubated with CJD3^{MV1} brain homogenate for 2 hours. After removal of the brain homogenate and multiple washes, the cells were either directly lyzed (0 hour) or maintained in culture in medium without brain homogenate for a further 6 or 24 hours. **A:** PrP aggregates recovered by NaPTA were left undigested or were digested with PK and were analyzed by Western blot analysis with the use of the anti-PrP monoclonal antibody 3F4. Molecular mass markers are shown on the left. **B:** Quantitation of the relative remaining amounts of total PrP aggregates (ie, both PK-resistant and -sensitive) versus PK-resistant PrP aggregates in the cells 6 and 24 hours after removal of brain homogenate. Light gray bars, PK-resistant PrP; dark gray bars, total PrP. **C:** Quantitation of the relative amounts of PK-resistant and -sensitive PrP aggregates present within the total PrP aggregates 6 and 24 hours after removal of brain homogenate from the cells. No statistical difference was found in the amount of PK-resistant and -sensitive PrP aggregates compared with the starting brain homogenate ($P = 0.83$, one-way analysis of variance with Dunnett's post test). Gray bars, PK-sensitive PrP; black bars, PK-resistant PrP. Values are expressed as means ± SEM (**B** and **C**). $n = 4$ experiments (**B** and **C**).

To determine whether the ratio of PK-sensitive to PK-resistant PrP^{Sc} aggregates changed over time as PrP^{Sc} was degraded by the cell, the total PrP aggregates associated with the cell (Figure 6B) were further divided into protease-sensitive and protease-resistant forms. The relative ratio of the two forms remained the same during 24 hours (Figure 6C). Thus, these results suggested that the degradation of PK-sensitive aggregated PrP^{Sc} occurred at a similar rate to that of PK-resistant aggregated PrP^{Sc}.

Discussion

One of the early events after exposure of cells to prion-infected brain homogenate is the cellular uptake of PrP^{Sc},[25,28–30] which is influenced by PrP^{Sc} particle size.[29,30] This active cellular uptake of PrP^{Sc} does not require PrP^{C} expression by host cells and is largely independent of prion strain.[25] Here, we have shown that both PK-sensitive and PK-resistant forms of aggregated PrP derived from sCJD brain interact similarly with human astrocytes for their uptake and degradation. Thus, our data suggest that both sPrP^{Sc} and rPrP^{Sc} are likely to be treated similarly by cells.

In general, when SVG cells were continuously exposed to brain homogenate, the predominant species of PrP taken up by the cell reflected that in the starting brain homogenate. Thus, in brain homogenates in which most PrP^{Sc} was aggregated but PK sensitive, most PrP^{Sc} in the cells was also aggregated but PK sensitive (Figure 3D). One exception to this observation was the cellular uptake of CJD1^{MM1} at 2 hours (Figure 3B). Although >90% of NaPTA-precipitated PrP aggregates were PK resistant in both CJD1^{MM1} brain homogenate and cells exposed to the homogenate for 8 to 48 hours, at 2 hours approximately 40% of the cell-associated aggregated PrP was protease sensitive. One possible explanation for this result may be the size of the PrP aggregate taken up by the cell. A specific population of PrP^{Sc} with smaller aggregate size can be preferentially taken up by cells during the early stages of prion infection,[30] and sPrP^{Sc} is known to be smaller in size than rPrP^{Sc}.[17,18] Thus, the increased amount of PK-sensitive PrP^{Sc} associated with the SVG astrocyte cells at 2 hours could be indicative of PK-sensitive, smaller forms of aggregated PrP^{Sc} being taken up more rapidly by the cells.

Our data also suggest that after uptake by astrocyte cells, PK-sensitive PrP^{Sc} aggregates from sCJD brains are degraded similarly to PK-resistant PrP^{Sc} aggregates. This result was somewhat unexpected because PrP^{Sc} is thought to be degraded by lysosomal proteases in cells,[31–33] and the two forms of aggregated PrP differ in their PK resistance. Although it is possible that the degradation kinetics of the two forms of PrP^{Sc} could still differ between the two time points we investigated (6 and 24 hours) (Figure 6), the observation that the ratio of PK-sensitive to PK-resistant PrP aggregates did not change during 24 hours (Figure 6C) suggests that this is unlikely. Furthermore, consistent with the *in vitro* data the clearance rate of sPrP^{Sc} and rPrP^{Sc} in scrapie-infected mice was also reported to be similar.[34] The lack of difference in the kinetics of cellular degradation between the two forms of PrP aggregate may be related to partial unfolding within the acidic cellular compartments to which PrP^{Sc} migrates after cellular uptake.[31,32,35,36] Low pH in the late endosomal/lysosomal cellular compartments may lead to the partial denaturation of aggregated PrP and the loss of structural motifs which likely confer differential proteolytic resistance. Alternatively, if there were nonprotein

cofactors acting as a molecular scaffold which contributed to the protease resistance of aggregated PrP,[37–39] partial denaturation in an acidic compartment might enable cellular enzymes to degrade them, thus leading to an increased susceptibility to proteolysis. In either case, cellular PK-resistant aggregates would become more protease sensitive and thus be degraded more rapidly than might be predicted from their biochemical properties.

It remains unclear how $sPrP^{Sc}$ and $rPrP^{Sc}$ are related metabolically in prion-infected brains, but previous studies have suggested that $sPrP^{Sc}$ may be an intermediate product in the formation of $rPrP^{Sc}$, a degradation product of $rPrP^{Sc}$, or the result of an alternative misfolding pathway.[17,19] Our data, indicating that the ratio of protease-resistant PrP^{Sc} to protease-sensitive PrP^{Sc} remains constant during PrP^{Sc} degradation in astrocyte cells, suggest an additional possibility that $sPrP^{Sc}$ and $rPrP^{Sc}$ can both be present in a single PrP^{Sc} aggregate. This could explain why $sPrP^{Sc}$ appears to have many of the properties of $rPrP^{Sc}$, including the ability to convert PrP^{C}[18,19] and to trigger prion infection *in vivo*.[20] Aggregated PrP^{Sc} grows by recruiting PrP^{C} and causing its conformational conversion into PrP^{Sc}. Protease-sensitive PrP^{Sc} could be the result of either incomplete conversion of the PrP^{C} associated with the aggregate to PrP^{Sc} or the location of a particular PrP^{Sc} molecule within the growing aggregate. Thus, $sPrP^{Sc}$ may represent a PrP^{Sc} molecule that is either not yet mature enough to become fully PK resistant or that is more easily accessible to proteases because of its presence in a less-protected part of the aggregate.

Alternatively, it is also possible that protease-sensitive and protease-resistant forms of PrP^{Sc} exist as separate populations in brain homogenate but coaggregate in specific cellular compartments (eg, early endosomes) after uptake by the cell. This would be consistent with data indicating that, in prion-infected hamsters, $sPrP^{Sc}$ and $rPrP^{Sc}$ can be isolated separately.[18,20] However, in the context of the well-known heterogeneity of PrP^{Sc}, it is certainly plausible that aggregates containing either $sPrP^{Sc}$ or $rPrP^{Sc}$ and mixed aggregates which differ for their ratio of $sPrP^{Sc}$ to $rPrP^{Sc}$ could coexist *in vivo*.

Given that $sPrP^{Sc}$ induces a prion disease in hamsters,[20] potentially influences the clinical duration of sCJD,[21] and converts PrP^{C} to $rPrP^{Sc}$,[18,19] it may play an active role in prion pathogenesis. Moreover, a role for astrocytes in prion pathogenesis is consistent with murine transmission studies in which neurotoxicity occurs even when PrP expression was restricted to astrocytes.[40,41] We have shown that protease-sensitive and protease-resistant PrP^{Sc} molecules are taken up and degraded by astrocyte cells similarly. These data suggest that $sPrP^{Sc}$ may be trafficked along similar pathways as $rPrP^{Sc}$. Thus, it would have ample opportunity to interact with and convert PrP^{C} to PrP^{Sc}. Our results are therefore consistent with the hypothesis that $sPrP^{Sc}$ would be available to potentially influence some aspects of prion pathogenesis[21] in both neurons and astrocytes.

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