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Protease-Resistant Human Prion Protein and Ferritin Are Cotransported across Caco-2 Epithelial Cells: Implications for Species Barrier in Prion Uptake from the Intestine

Ravi Shankar Mishra,* Subhabrata Basu,* Yaping Gu, Xiuo Luo, Wen-Quan Zou, Richa Mishra, Ruliang Li, Shu G. Chen, Pierluigi Gambetti, Hisashi Fujioka, and Neena Singh
Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

Foodborne transmission of bovine spongiform encephalopathy (BSE) to humans as variant Creutzfeldt–Jakob disease (CJD) has affected over 100 individuals, and probably millions of others have been exposed to BSE-contaminated food substances. Despite these obvious public health concerns, surprisingly little is known about the mechanism by which PrP–scrapie (PrPSc), the most reliable surrogate marker of infection in BSE-contaminated food, crosses the human intestinal epithelial cell barrier. Here we show that digestive enzyme (DE) treatment of sporadic CJD brain homogenate generates a C-terminal fragment similar to the proteinase K-resistant PrPSc core of 27–30 kDa implicated in prion disease transmission and pathogenesis. Notably, DE treatment results in a PrPSc–protein complex that is avidly transcytosed in vesicular structures across an in vitro model of the human intestinal epithelial cell barrier, regardless of the amount of endogenous PrP expression. Unexpectedly, PrPSc is cotransported with ferritin, a prominent component of the DE-treated PrPSc–protein complex. The transport of PrPSc–ferritin is sensitive to low temperature, brefeldin-A, and nocodazole treatment and is inhibited by excess free ferritin, implicating a receptor- or transporter-mediated pathway. Because ferritin shares considerable homology across species, these data suggest that PrPSc–associated proteins, in particular ferritin, may facilitate PrPSc uptake in the intestine from distant species, leading to a carrier state in humans.

Key words: prion infection; subclinical infection; PrP transport; new variant CJD; ferritin; epithelial cell barrier; Caco-2

Introduction

The transmission of sheep scrapie to cattle as bovine spongiform encephalopathy (BSE) and its onward transmission to humans as variant Creutzfeldt–Jakob disease (vCJD) attests to the remarkably persistent and permeable nature of prions or PrP–scrapie (PrPSc) across species barriers (Hill et al., 1998; Collinge, 1999; Taylor, 2002). The BSE epidemic is far from over despite the concerted efforts of national, industrial, and regulatory agencies across the world. An emerging threat is the continual spread of chronic wasting disease in the deer and elk population in the United States and the uncertainties regarding its transmission to livestock and humans (Miller and Williams, 2003). As the sources of PrPSc–contaminated food products continue to increase, it has become increasingly critical to understand the mechanism by which PrPSc, a protein with a protease-resistant core of 27–30 kDa and a major, if not the only, component of prion infectivity (Prusiner, 1998), maneuvers its way across the impermeable and highly selective epithelial barrier of the human intestinal tract.

Retrospective examination of vCJD patients and animal models challenged orally with BSE-infected tissue show accumulation of PrPSc in the Peyer’s patches, lymphoid tissue lining the gastrointestinal (GI) tract, and peripheral and enteric nervous systems (Bons et al., 1999; Beekes and McBride, 2000; Foster et al., 2001; McBride et al., 2001; Nicotera, 2001; Haik et al., 2003; Aguzzi and Polymenidou, 2004). Uptake of PrPSc from the lumen of the intestine is thought to be mediated by intestinal dendritic cells and M-cells lining the mucosa, after which it undergoes replication in the gut-associated lymphoid tissue. Subsequent transport to the CNS probably occurs along peripheral nerves (Heppner et al., 2001; Huang et al., 2002; Aguzzi and Polymenidou, 2004). However, a recent report demonstrating the absence of prion infectivity in μMT and RAG1−/− mice orally challenged with prions despite the presence of M-cells suggests that PrPSc transport across the intestinal epithelial barrier is not limited to M-cells and that additional pathways must exist (Prinz et al., 2003).

Thus, to fully understand the mechanism of PrPSc uptake from contaminated food by the intestinal epithelial cells, we investigated the transport of human PrPSc from sporadic CJD brain tissue (sCJD–PrPSc) across a monolayer of Caco-2 cells with tight junctions, representing an in vitro model of the human intestinal epithelial cell barrier (Pinto et al., 1983). Here we show that pre-
Treatment of sCJD brain homogenate with digestive enzymes (DEs), in particular stomach pepsin, generates a protease-resistant C-terminal fragment similar to the proteinase K (PK)-resistant core of PrPSc (PrP27–30) implicated in the transmission and pathogenesis of prion disorders (Prusiner, 1998). Unexpectedly, both PK and DE treatments generate a PrPSc–protein complex that includes ferritin as a major component, and the PrPSc– ferritin complex is cotransported across Caco-2 cells in vesicular structures. The transport of PrPSc–ferritin complex is inhibited by excess free ferritin, low temperature, and by treatment with brefeldin-A or nocodazole, implicating a receptor- or transporter-mediated transcytotic path across Caco-2 cells. These data provide insight into the cellular mechanisms by which PrPSc ingested with contaminated food crosses the intestinal epithelium and the possibility of devising practical methods for blocking its uptake.

Materials and Methods

Materials and chemicals. Normal human brain tissue was obtained from frozen samples from a 61-year-old female and diseased tissue from a 66-year-old male with a confirmed diagnosis of sCJD. Human colon carcinoma cell lines Caco-2 (C2BBe1) (Peterson et al., 1992) and HT-29 were obtained from American Type Culture Collection (Manassas, VA). The following anti-PrP antibodies were used in this study: 3F4 (residues 109 and 112; Signet Laboratories, Dedham, MA), 8H4 (residues 175–185; obtained from our facility), 8B4 (residues 37–44; obtained from our facility), and 6H4 (residues 145–152; Prionics). The antibody against the tight junction protein zonula occludens-1 (ZO-1) was purchased from Zymed (San Francisco, CA). Polyclonal anti-ferritin antibody was obtained from Sigma (St. Louis, MO). RTIC- and FITC-labeled secondary antibodies were obtained from Southern Biotechnology (Birmingham, AL). Sulfo-NHS-biotin and streptavidin–Texas Red were obtained from Pierce (Rockford, IL). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA). Pure human liver and spleen ferritin and all other chemicals were obtained from Sigma.

Cell culture and preparation of epithelial cell monolayers. Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum in a 10% CO2 atmosphere and passaged weekly. For preparing monolayers, cells from a confluent flask were resuspended in DMEM at a concentration of 2 × 10^5 cells/ml and added to the apical (AP) chamber of polylysine-coated glass coverslips. The filters were placed in the BL chamber for the duration of the experiment. For PK treatment, the homogenate was supplemented with lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 100 mM NaCl, and 10 mM EDTA in 20 mM Tris·HCl, pH 7.4) and treated with 50 μg/ml PK at 37°C for 1 hr. The reaction was terminated with 4 mM PMSF and a mixture of protease inhibitors (described above), and the homogenate was frozen at −70°C for additional use.

For immunoprecipitation, untreated or PK- or DE-treated NHs and sCJDHs were centrifuged at 3000 × g for 15 min at 4°C, and the supernatant was subjected to immunoprecipitation with either anti-ferritin or anti-PrP antibodies 6H4 or 8H4, as described previously (Mishra et al., 2002). The protein complexes were eluted from protein A beads with low pH glycine buffer, the pH was adjusted, and small aliquots of immunoprecipitated samples were frozen for additional use. Transport studies were performed in duplicate with each of these samples simultaneously to minimize experimental error.

Purification of PrPSc from sCJD brain homogenate. For the isolation of purified PrPSc, 0.3 g of sCJD brain sample was homogenized in PBS to yield a 10% homogenate and biotinylated with 1 mg/ml sulfo-NHS-biotin (Pierce) overnight at 4°C. Excess biotin was quenched with 50 mM glycine and by washing three times with PBS in a centricron with a 3 kDa cutoff. Biotinylated CJDH was supplemented with an equal volume of 2× lysis buffer and centrifuged at 890 × g for 10 min to pellet large aggregates (P1). The recovered supernatant (S1) was ultracentrifugated at 100,000 × g for 1 hr at 4°C to obtain the pellet P2, which was resuspended again in lysis buffer and recentrifuged to obtain the pellet P3. At this stage, the pellet was redissolved in TNSS buffer (10 mM Tris, 1 mM EDTA, 1 mM DTT, 1% sarcosyl, and 135 mM NaCl) and treated with 50 μg/ml PK at 37°C for 1 hr. The reaction was stopped with 4 mM PMSF and the mixture of protease inhibitors and subjected to an additional round of ultracentrifugation at 200,000 × g for 2 hr to obtain the PrPSc-rich pellet fraction P4. The pellet P4 was resuspended again in TNSS buffer and recentrifuged at the same speed to obtain sequentially pellet fractions P5 and P6. In parallel, normal brain tissue was subjected to a similar treatment and used as a control for transport and binding experiments. The pellet obtained from both sCJD and normal brain samples were suspended in 100 μl of PBS and sonicated with an equal volume of 20% purified human brain total lipid extract obtained from Avanti Polar Lipids (Alabaster, AL) to yield a 10% lipid–protein mixture. The resulting NH Pellet-, and CJDH Pellet–brain lipid suspensions were diluted in PBS containing 1% BSA and used for binding and competition experiments.

Measurement of PrPSc transport. In a typical experiment, monolayers of Caco-2 cells were washed with serum-free medium, and 20 μl of sample dissolved in 1 ml of serum-free medium was added to the AP chamber. The sample consisted of NH or CJDH that was untreated, PK or DE treated, or DE treated and mixed with 10 μM PrP peptide 106–126. The inserts were placed in a 6-well dish containing 1.2 ml of serum-free medium and incubated overnight at 37°C. Subsequently, AP and BL media samples were collected and centrifuged to pellet cell debris, and proteins from the supernatant were isolated by cold methanol precipitation. For preparation of cell lysate, cells on monolayers were treated with lysis buffer, and proteins were precipitated as above. All samples were boiled in sample buffer, resolved by SDS-PAGE, electrophoresed to a polyvinylidene difluoride (PVDF) membrane, and probed with specific antibodies.

Quantitative analysis was performed by measuring the total raw density of PrPSc bands in the AP and BL medium from duplicate samples. Each experiment was repeated five to eight times, and the statistical significance was evaluated by Student’s t test.
Silver staining of total proteins. After SDS-PAGE, proteins were stained with the silver staining kit according to the instructions provided by the manufacturer (Bio-Rad, Hercules, CA).

Competitive inhibition experiments. Caco-2 monolayers cultured on filter inserts were cut out with a sharp scalpel and inverted with the cell side down on 16 μl of NH or CJDH. A suspension mixed in 84 μl of PBS containing 1% BSA or 0.05, 1.0, and 1.5 μg/ml human spleen or liver ferritin dissolved in the same buffer. After a 30 min incubation on ice, filters were removed and washed gently in ice-cold PBS. Subsequent incubation with the specified sample was performed similarly. The cells were then fixed with 4% paraformaldehyde, immunostained as such on filters, and mounted with the cell side facing the coverslip for confocal microscopy.

Immunostaining and confocal microscopy. Cells were cultured on poly-d-lysine-coated glass coverslips or on transparent Transwell filters. After a particular experimental treatment, cells were fixed and processed for staining or first permeabilized with Triton X-100 and reacted with one of the following primary antibodies: monoclonal anti-PrP 8H4 (1:20), polyclonal rabbit anti-ferritin (1:20), or polyclonal anti-ZO-1 (1:20), followed by RITC- or FITC-conjugated appropriate (mouse or rabbit) secondary antibodies as described previously (Gu et al., 2003a,b). Streptavidin–Texas Red was used at a concentration of 1:40. Immunostained cells were mounted in gel mount and observed using a laser-scanning confocal microscope (Bio-Rad MRC 600). Horizontal sections were imaged using a 60 × objective, and a magnification of 1.0 or 2.5 at different depths beginning from the top of the cells until the filter pores were visible. Vertical images were captured similarly using one filter at a time (green or red). Selected samples were reexamined and imaged using the LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

Electron microscopy (transmission electron microscopy). Caco-2 cells on filter inserts were exposed to 8H4-immunoprecipitated CJDH–PrPSc for 2 hr and fixed in a buffer containing glutaraldehyde (2.5%), paraformaldehyde (2%), and sucrose (4%) in phosphate buffer (0.05 M; pH 7.4) for 2 hr. Cell monolayer on the filter was cut out of the insert and postfixed with 1% osmium tetroxide for 1 hr, followed by 30 min of en bloc staining with 1% aqueous uranyl acetate. Cells were then dehydrated in ascending concentrations of ethanol and embedded in Epon 812. Ultrathin sections were treated with 1% periodic acid for 4 min and stained with 2% uranyl acetate and lead citrate in 50% methanol. Processing of the 8H4-immunoprecipitated material was similar, with the modification that the sample was fixed, osmicated and treated with uranyl acetate solution, and embedded in agar by centrifugation on a 1.5% agar block in an Eppendorf tube. The Eppendorf tube was then cut open with a blade, and the pellet embedded in agar was dehydrated and processed as above. All samples were examined using a CEM902 electron microscope (Zeiss).

Results

Human sCJD–PrPSc is partially proteolyzed by DEs

PrPSc ingested with contaminated meat is exposed to the harsh environment within the GI tract before uptake by the lining epithelium. During this process, the effect of DE and variable pH on the structure and stability of PrPSc and PrPSc is not known. To address this question, samples of NH and CJDH were subjected to sequential treatment with DE to simulate the environment within the GI tract before uptake by the lining epithelium before DE treatment.

In a typical experiment, 20 μl of NH or CJDH that had been pretreated with conventional PK treatment by subjecting mock-treated, PK-treated, and DE-treated samples to Western blot analysis with anti-PrP antibody 3F4. Mock-treated NH reveals the diglycosylated, monoglycosylated, and undiglycosylated forms of PrPSc migrating at 35–37, 28–30, and 27 kDa respectively (Fig. 1A, lane 1). Similar glycoforms are detected in mock-treated CJDH, although the ratio of the three glycoforms is altered (Fig. 1A, lane 4). Treatment with PK or DE results in complete proteolysis of PrPSc in NH (Fig. 1A, lanes 2, 3), whereas CJDH samples show faster migrating forms of 27–30 and 19 kDa, consistent with the migration of infectious and pathogenic PK-resistant PrPSc (Fig. 1A, lanes 5, 6). Although this outcome is expected after PK treatment of CJDH, the generation of similar glycoforms with DE is noteworthy, suggesting comparable cleavage of PrPSc by the two procedures. Additional confirmation of DE-mediated cleavage of PrPSc was obtained by reblotting PK- and DE-treated CJDH samples with antibodies specific to the N or C terminus of PrP. As expected, there was no immunoreaction with the N-terminal antibody 8B4 and strong reactivity with C-terminal antibody 8H4 (data not shown). Evaluation of sCJD brain homogenate after treatment with individual DEs revealed that the cleavage of PrPSc is mediated by pepsin at pH 2.0 (data not shown).

Quantitative estimation of the above results shows that 2 and 3% of PrP in NH samples and 68 and 75% of PrP in CJDH samples resist PK and DE treatment, respectively (Fig. 1B). Thus, by the time ingested PrPSc-contaminated meat reaches the intestine, almost all of PrPSc is proteolyzed, and PrPSc is converted to the protease-resistant C-terminal core of 27–30 kDa.

DE-treated sCJD–PrPSc is transported across Caco-2 epithelial cells

The transport of PrPSc in NH and CJDH across intestinal epithelial cells was assessed in an in vitro model comprising Caco-2 cell monolayers with tight junctions (Pinto et al., 1983). For all experiments, Caco-2 monolayers exhibiting a TEER of >400 Ω·cm² and 3H-inulin transport from the AP chamber to the BL chamber of Caco-2 monolayers in serum-free medium and incubated overnight at 37°C. Subsequently, medium was collected from the AP and BL chambers, and methanol–precipitated proteins from the media and cell lysate samples were fractionated by SDS-PAGE, transblotted to a PVDF membrane, and probed with 3F4. In the NH sample, practically all of the added PrP was recovered from the AP medium, indicating negligible transport to the BL chamber (Fig. 2A, lanes 1–3). As expected, NH-PK and NH-DE samples show barely detectable PrP signal in the AP or
BL samples (Fig. 2A, lanes 4–7). However, PrP106–126 (toxic peptide) is easily detected in the AP medium of NH-DIE plus toxic peptide sample, ruling out experimental error in the detection of any leftover PrP (Fig. 2A, lane 8, arrowhead). In contrast, PrP in CJDH and protease-resistant PrPSc in PK- and DE-treated CJDH samples are transported to the BL chamber (Fig. 2B, lanes 10–16). Surprisingly, PrPSc in DE-treated CJDH is transported more efficiently than from the PK-treated sample (Fig. 2B, lanes 10–18). The PrP106–126 peptide mixed with CJDH-DE is not transported, although a bold PrPSc signal is detected in the BL chamber of CJDH-DE plus toxic peptide sample (Fig. 2B, lanes 17, 18, arrowhead). No PrP signal was detected in any of the Caco-2 lysate samples (data not shown).

Quantification of the PrP signal in the AP and BL chambers of monolayers exposed to NH or CJDH and the percentage of transport of PrP in CJDH from the AP to the BL chamber are shown in Table 1. A prominent PrP signal is detected in the AP and BL chambers of monolayers exposed to similarly treated CJDH samples (lane numbers correspond to samples in A and B). C, Quantitative estimation shows transport of 14, 8.5, and 42% of PrP from the AP chamber to the BL chamber from mock-treated, PK-treated, and DE-treated CJDH, respectively. Transport of ^3H-inulin was <0.01%/cm^2/min before and after the completion of each experiment. The error bar represents the mean ± SD of four experiments. p < 0.01.

After an overnight incubation, AP and BL media were analyzed (Fig. 2C, lanes 2, 3). In the PK- and DE-treated NH samples, as expected, barely any PrP is detected in the AP or BL chambers (Fig. 2C, lanes 4–7). In contrast, a significant proportion of PrPSc from the CJDH samples is transported from the AP to the BL chambers (Fig. 2C, lanes 11–16), representing a transport of 14, 8.5, and 42% of PrPSc from mock-treated, PK-treated, and DE-treated CJDH, respectively (Fig. 2D, lanes 11–16). The transport of ^3H-inulin across the same monolayer is <0.01%/cm^2/min in 1 hr at 37°C (Fig. 2D), confirming the integrity of tight junctions under these experimental conditions.

Figure 2C and D, respectively. In the mock-treated NH sample, almost all PrP is recovered from the AP chamber, with insignificant transport to the BL chamber (Fig. 2C, lanes 2, 3). In the PK- and DE-treated NH samples, as expected, barely any PrP is detected in the AP or BL chambers (Fig. 2C, lanes 4–7). In contrast, a significant proportion of PrPSc from the CJDH samples is transported from the AP to the BL chambers (Fig. 2C, lanes 11–16), representing a transport of 14, 8.5, and 42% of PrPSc from mock-treated, PK-treated, and DE-treated CJDH, respectively (Fig. 2D, lanes 11–16). The transport of ^3H-inulin across the same monolayer is <0.01%/cm^2/min in 1 hr at 37°C (Fig. 2D), confirming the integrity of tight junctions under these experimental conditions.

Overexpression of PrP^C does not alter the transport of sCJD–PrPSc across Caco-2 cells
To evaluate whether the level of PrP^C expression on Caco-2 cells influences PrPSc uptake or transport, Caco-2 cells were transfected with a plasmid encoding human PrP^C, and the percentage of increase in PrPSc expression was estimated. Immunoblotting of cell lysates prepared from human neuroblastoma, nontransfected Caco-2, and PrP^C-transfected Caco-2 (Caco-2^Prp) cells with 3F4 shows a 2.5-fold increase in PrPSc expression by Caco-2^Prp cells compared with nontransfected Caco-2 cells and 1.25 times that of M17 neuroblastoma cells (Fig. 3A, lanes 1–3, B).

The influence of increased PrP^C expression on PrPSc transport was estimated by isolating a crude fraction of PrPSc from PK-treated CJDH to avoid the influence of membrane in mediating PrPSc transport (Baron and Caughey, 2003). Accordingly, 20 μl of PK-treated CJDH was methanol precipitated, and the pellet was resuspended in PBS and added to the AP chamber of Caco-2 and Caco-2^Prp monolayers.

Protease-resistant human sCJD–PrPSc is associated with ferritin
We next attempted to purify PrPSc from sCJDH to determine the impact of other molecules or proteins on its transport across
Caco-2 cells. Thus, sCJDH was subjected to PK treatment and repeated rounds of ultracentrifugation as described in Materials and Methods. The clarified supernatant from CJDH (S1), the supernatant and pellet fractions after the first round of ultracentrifugation (S2 and P2, respectively), and four subsequent rounds of ultracentrifugation (S3–S6 and P3–P6) were precipitated with cold methanol, fractionated by SDS-PAGE, and transblotted. Probing with 3F4 reveals PK-resistant glycoforms of PrP\(^S\)c representing the N-terminal truncated diglycosylated, monoglycosylated, and unglycosylated forms migrating at 29 and 30, 22–25, and 19 kDa, respectively, in the S1 fraction (Fig. 4A, lane 1). After the first round of ultracentrifugation, \(\sim 40\%\) of PrP\(^S\)c fractionates in the supernatant fraction (S2), and \(\sim 60\%\) is detected in the pellet (P2) (Fig. 4A, lanes 2, 3). In subsequent rounds, all of the PrP\(^S\)c is detected in the pellet fractions (P3–P6) (Fig. 4A, lanes 5, 7, 9, 11).

To assess the purity of PrP\(^S\)c recovered in the P6 fraction, the sample was deglycosylated with PNGase-F, fractionated by SDS-PAGE, and visualized by silver staining. In the untreated sample, bands corresponding to the diglycosylated, monoglycosylated, and unglycosylated forms of PrP\(^S\)c are identified as in Figure 4A (Fig. 4B, lane 1). After deglycosylation, PrP\(^S\)c glycoforms collapse to 19 kDa (Fig. 4B, lane 2, arrow). The band marked with an asterisk represents the added PNGase (Fig. 4B, lane 2). Sequencing of the 20 kDa band confirmed its identity as a mixture of heavy (H) and light (L) chains of ferritin. Additional verification was obtained by reproducing the membrane in Figure 4A with anti-ferritin antibody. Strong immunoreaction is detected with the 20 kDa band, confirming its identity as ferritin (Fig. 4C, lanes 1–11). It is remarkable that ferritin resists PK treatment and persistently pellets with PrP\(^S\)c.

The above results argue that either PrP\(^S\)c and ferritin happen to cosediment or the two proteins form a complex with each other, perhaps through ionic or hydrophobic interactions. To distinguish between these possibilities, the P6 pellet fraction was treated with NaCl varying in concentration from 0.1 to 1.0 M, and ferritin was eluted using DEAE-cellulose chromatography. Immunoblotting of the eluted fractions with 3F4 and anti-ferritin antibody shows complete elution of ferritin at 0.4 M NaCl (Fig. 4D, bottom, lanes 1–6). Almost all of the PrP\(^S\)c is retained in the column and is barely detected in the eluate (Fig. 4D, top, lanes 1–6).

Thus, PrP\(^S\)c and ferritin in the sCJD brain homogenate form a complex that is resistant to dissociation with low concentrations of salt. Whether this interaction occurs in the brain in vivo or after homogenization of brain tissue is unclear from our data.

Because it is unlikely that an aggregated and insoluble PrP\(^S\)c–ferritin complex would be transported across the epithelial cell barrier, we focused our additional studies on the PK-resistant but detergent-soluble species of PrP\(^S\)c that is known to be infectious and can be immunoprecipitated with anti-PrP antibodies 8H4 and 6H4 (Safar et al., 1998; Paramithiotis et al., 2003; Pan et al., 2001). To determine whether protease-resistant, detergent-soluble PrP\(^S\)c is similarly associated with ferritin, mock-treated and DE-treated NH and CJDH were clarified by centrifugation at 3000 \(\times\) g and subjected to immunoprecipitation with either anti-ferritin or anti-PrP antibody 8H4. Immune complexes were collected with protein A beads and washed, and eluted proteins were analyzed by immunoblotting with 3F4 or anti-ferritin antibodies. In samples immunoprecipitated with anti-ferritin and probed with 3F4, minimal PrP signal is detected in NH and NH-DE samples (Fig. 5A, lanes 1, 2). In contrast, surprisingly large
amounts of 3F4-immunoreactive PrPSc from CJDH-DE and a small amount from CJDH sample coimmunoprecipitates with anti-ferritin (Fig. 5A, lanes 3, 4). Reprobing of the same membrane with anti-ferritin reveals the H and L chains of ferritin migrating at 21 and 20 kDa, respectively (Fig. 5A, lanes 5–8). In the CJDH-DE sample, additional slower migrating bands that react strongly with anti-ferritin antibody are detected (Fig. 5A, lane 8). Their identity is not clear at present. Immunoprecipitation of CJDH-DE with 8H4, followed by probing with 3F4 or anti-ferritin antibodies, shows similar association of PrPSc with ferritin (Fig. 5A, lanes 9, 10). The apparent difference in the amount of PrPSc and ferritin that coimmunoprecipitate with anti-ferritin versus 8H4 is probably attributable to the nature of the specific antibodies (N. Morel et al., 2004). DE treatment partially hydrolyzes the H chain of ferritin, which comigrates with the L chain at 20 kDa (Fig. 5A, lane 5 vs 6, 7 vs 8).

Silver staining of anti-ferritin- and 8H4-immunoprecipitated proteins from DE-treated NH and CJDH shows bands comigrating with ferritin at 20 kDa and several unidentified proteins (Fig. 5A, lanes 11–14). No PrP was immunoprecipitated in the absence of primary antibody from either mock-treated or DE-treated NH or CJDH, confirming that PrPSc does not bind nonspecifically to protein A beads (Fig. 5B).

**sCJD–PrPSc** is cotransported with ferritin across Caco-2 cells

To determine whether PrPSc is transported across Caco-2 cells in association with ferritin, 20 μl of CJDH-DE in serum-free medium was added to the AP chamber of filter inserts containing Caco-2 cell monolayers and incubated for 2 hr at 37°C. At the end of the incubation, monolayers were cut into two pieces: one half was immunostained for PrP and ferritin, and the other half was immunostained for the tight junction protein ZO-1. Transport of PrP and ferritin was checked by capturing horizontal confocal images at different depths as depicted in Figure 6A and by taking vertical images.

Staining for PrP (green) and ferritin (red) at level I shows colocalization of PrP and ferritin (Fig. 6B, panels 1–3, arrows) and limited reactivity for PrP alone (Fig. 6B, panels 1, 3, arrowheads). Similar images captured at level II (at the level of the filter pores) also show colocalization of PrP (green) and ferritin (red) (Fig. 6B, panels 4–6, arrows). Immunostaining for the tight junction protein ZO-1 (green) reveals intact tight junctions throughout the Caco-2 monolayer (Fig. 6B, panel 7). A vertical image through the same cells shows colocalization of PrP (green) and ferritin (red) at the AP and BL membranes (levels I and II) (Fig. 6B, panels 8–10). Transport of both PrPSc and ferritin was significantly inhibited by incubation at 18°C and by pretreatment of the cells for 2 hr with brefeldin-A (3.5 μM) or nocodazole (33 μM), implicating a transcytotic process (data not shown).

**sCJD–PrPSc** remains associated with ferritin after transcytosis

To evaluate whether the PrPSc–ferritin complex remains intact after transcytosis across Caco-2 cells, filter inserts containing Caco-2 cell monolayers were placed in a 12-well dish containing M17 neuroblastoma cells cultured on glass coverslips in the BL chamber (Fig. 6A, diagram). Subsequently, 20 μl of CJDH-DE or biotinylated CJDH-DE was added to the AP chamber. The biotinylated sample was used to distinguish added PrP and ferritin from endogenous proteins expressed by M17 cells. After an overnight incubation, transcytosed PrPSc and ferritin that had been subsequently endocytosed by M17 cells in the BL chamber were detected by immunostaining (Fig. 7A). The presence of tight junctions in Caco-2 cell monolayers was confirmed by immuno-
that the PrPSc–ferritin complex is endocy-
tised together by Caco-2 cells.

The binding of sCJD–PrPSc to Caco-2
the BL chamber, where it is endocytosed
icant proportion is transcytosed intact to
rations were used: (1) pure human liver ferritin; (2) pure human
ferritin-binding sites. Two different PrPSc preparations were
used for this purpose: (1) partially denatured biotin-tagged PrPSc
added to a monolayer of Caco-2
cells, the PrPSc–ferritin complex is seen in
small and large phagocytic vesicles enclosed
by a single membrane with the fibrillar ma-
material intact within these vesicles (Fig. 7B,
top). Groups of these vesicles are subse-
quently transported out from the BL mem-
brane and are seen within the pore of the
Transwell membrane (Fig. 7B, bottom).

Together, the above data demonstrate
that the PrPSc–ferritin complex is endocy-
tosed together by Caco-2 cells and a signifi-
cant proportion is transcytosed intact to
the BL chamber, where it is endocytosed
again by M17 cells.

The binding of sCJD–PrPSc to Caco-2
cells is partially inhibited by
excess ferritin
The persistent association of PrPSc with
ferritin before and after transcytosis led us
to investigate whether ferritin acts as a fa-
cilitator or a mediator of PrPSc transport
across Caco-2 cells. Accordingly, an at-
tempt was made to competitively inhibit
the binding of PrPSc by preincubating
Caco-2 cells with increasing amounts of
purified ferritin to saturate available ferritin-binding sites. Two different PrPSc preparations were
used for this purpose: (1) partially denatured biotin-tagged PrPSc
isolated from CJDH that copurifies with ferritin after ultra-
centrifugation; and (2) biotin-tagged PrPSc–ferritin in its native con-
formation in CJDH-DE. For competition, three different prepa-
ations were used: (1) pure human liver ferritin; (2) pure human
spleen ferritin; and (3) brain ferritin purified from NH
(NHPellet).

Biotin-tagged PrPSc was purified by subjecting biotinylated
CJDH to PK treatment and repeated rounds of ultracentrifuga-
tion, as in Figure 4. A sample from biotinylated NH was subjected
to similar treatment, and the resulting pellet fractions from NH
(NH Pellet) and CJDH (CJDH Pellet) were evaluated by Western
blotting and silver staining. As expected, immunoblotting with
3F4 shows no reactivity with the NH sample and strong reactivity
with N-terminally truncated PK-resistant PrPSc bands in the
CJDH sample (Fig. 8A, lanes 1, 2). Reprobing of the membrane
with anti-ferritin antibody shows the presence of ferritin in both
NH and CJDH samples (lanes 5, 7). The H chain comigrates with the L chain at 20 kDa after DE treatment of NH and CJDH (lanes 6, 8). The identity
of additional ferritin-immunoactive bands in the CJDH-DE sample is unclear (lane 8, *). I.P. of CJDH-DE with 8H4, followed by
probing with 3F4, shows PrPSc bands similar to the ones detected with anti-ferritin I.P. (compare lanes 9, 4). Reprobing
of the same membrane with anti-ferritin shows the H and L chains of ferritin migrating at 21 and 20 kDa in both NH and CJDH
samples (lanes 5, 7). The H chain comigrates with the L chain at 20 kDa after DE treatment of NH and CJDH (lanes 6, 8). The identity
of additional ferritin-immunoactive bands in the CJDH-DE sample is unclear (lane 8, *). I.P. of CJDH-DE with 8H4, followed by
probing with 3F4, shows PrPSc bands similar to the ones detected with anti-ferritin I.P. (compare lanes 9, 4). Reprobing
with anti-ferritin reveals the 20 kDa ferritin band (lane 10). Silver staining of proteins immunoprecipitated with anti-ferritin or 8H4
shows bands comigrating with ferritin at 20 kDa and several other minor species (lanes 11–14). Note that the IgG band in 8H4
immunoprecipitates is negligible because the antibody was conjugated to protein A beads. A similar conjugation of anti-ferritin
reduced the amount of coimmunoprecipitated PrPSc significantly. B, No PrP bands are detected in the absence of primary antibody
either with protein A beads or BSA-coated protein A beads (lanes 1–8).

Figure 5. The PrPSc–ferritin complex coimmunoprecipitates with anti-ferritin and anti-PrP antibodies. Mock-treated or DE-
treated NH and CJDH samples were immunoprecipitated (I.P.) with anti-ferritin or 8H4 antibodies, and coimmunoprecipitated
proteins were detected by immunoblotting with specific antibodies. A, I.P. with anti-ferritin, followed by probing with 3F4, shows
no coimmunoprecipitation of PrPSc from NH and NH-DE samples (lanes 1, 2). In contrast, full-length PrPSc and a significantly
greater amount of protease-resistant PrPSc from CJDH and CJDH-DE immunoprecipitate with anti-ferritin (lanes 3, 4). Reprobing
of the membrane with anti-ferritin shows the H and L chains of ferritin migrating at 21 and 20 kDa in both NH and CJDH
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either with protein A beads or BSA-coated protein A beads (lanes 1–8).
ferritin, NH_Pellet and CJDH_Pellet fractions were resuspended in purified human brain lipids lacking all proteins and sonicated to obtain a homogeneous mixture. Subsequently, polarized monolayers of Caco-2 cells were exposed to increasing amounts of NH_Pellet and CJDH_Pellet—brain lipid mixture diluted in PBS containing 1% BSA for 30 min on ice and processed for staining with Texas Red–streptavidin. Remarkably, cells exposed to both NH_Pellet and CJDH_Pellet show significant binding as determined by biotin-specific reactivity (Fig. 8 B, panels 1, 2, −Ferritin). Under the experimental conditions used, 16 μl of NH_Pellet and CJDH_Pellet—brain lipid preparation gave a reproducible and specific signal of defined intensity and was used for competition experiments. Thus, cells were exposed to 0, 0.5, 1.0, and 1.5 μg/ml human spleen ferritin resuspended in PBS containing 1% BSA for 30 min on ice, washed, and reexposed to 16 μl of NH_Pellet and CJDH_Pellet for an additional 30 min on ice. The amount of streptavidin-reactive material bound to Caco-2 cells was determined by staining with Texas Red–streptavidin. Under these experimental conditions, 1.5 μg/ml pure spleen ferritin inhibits the binding of NH_Pellet and CJDH_Pellet by ~80%, as determined by comparing the mean fluorescence intensity in 20 different fields (Fig. 8 B, panels 3, 4, +Ferritin). Anti-ferritin- and PrP-specific antibodies were not used in this experimental setup because immunoreactivity is lost because of DTT treatment. The brain lipid used as vehicle did not show any reaction by itself (data not shown).

The binding of NH_Pellet that comprises only human brain ferritin and CJDH_Pellet that comprises partially denatured PrPSc and ferritin and the inhibition of this binding by purified spleen ferritin suggest strongly that the binding of the PrPSc–ferritin complex to Caco-2 cells is mediated by ferritin, not by PrPSc.

Similar competition experiments were performed with biotin–tagged PrPSc in CJDH–DE, a milieu in which it maintains reactivity to the anti-PrP antibody 8H4. The inhibition of PrPSc binding in the presence of saturating amounts of ferritin was assessed by double staining with 8H4 and Texas Red–streptavidin. Although significant inhibition (~85%) of PrPSc binding is observed in the presence of 1.5 μg/ml spleen ferritin, we did not observe a complete block (Fig. 8 C, panels 1–6, −Ferritin and +Ferritin). Similar results were obtained when liver ferritin was used as a competitive inhibitor (data not shown).

Our inability to demonstrate >80–85% inhibition of PrPSc binding despite high concentrations of free ferritin as a competitor led us to conclude that liver and spleen ferritin may not be the optimal inhibitors. Because ferritin in NH_Pellet is similar to the ferritin in CJDH–DE in terms of the source and method of preparation, we used NH_Pellet to saturate available ferritin-binding sites on Caco-2 cells before adding CJDH–DE. Thus, Caco-2 cells were exposed to 16 μl of NH_Pellet—brain lipid suspension for 30 min on ice, washed, and incubated for an additional 30 min on ice with 25 μl of CJDH–DE. The cells were then immunostained with 8H4 to detect bound PrPSc. Preincubation of the cells with 16 μl of NH_Pellet inhibited the binding of PrPSc by ~90% (Fig. 8 D, panels 1, 2, −NH_Pellet and +NH_Pellet).

Together, the above results suggest strongly that ferritin plays a significant role in the binding and transport of the PrPSc–ferritin complex across Caco-2 cells.

Discussion

This report provides insight into the pathway of PrPSc uptake and transport across intestinal epithelial cells. In particular, our data show that exposure of sCJD brain homogenate to DEs generates a C-terminal PrPSc core of 27–30 kDa that is transported across
Caco-2 cells in vesicular structures and that this process is not influenced by the level of endogenous PrPSc expression. Within these vesicles, PrPSc is associated with ferritin, a major component of the PrPSc–protein complex, and remains associated with ferritin after transcytosis. Because ferritin is normally absorbed from food and is abundantly present in a typical meat dish, these findings have important implications for prion uptake from contaminated food.

Using the well tested in vitro model for evaluating intestinal uptake of selected food nutrients (Cereijido et al., 1978; Pinto et al., 1983; Glahn et al., 1998), we show the resilience of PrPSc to DEs and the facilitative effect of such treatment on PrPSc uptake by Caco-2 cell monolayers. We noted that after treatment of CJDH with stomach pepsin, PrPSc underwent limited proteolysis and comigrated with the C-terminal PK-resistant core of PrPSc. Under similar conditions, PrPSc in the NH was completely hydrolyzed. Much to our surprise, DE-treated PrPSc was transported across Caco-2 cells four times more efficiently than PK-treated PrPSc. We believe that this effect is attributable to the chaotropic effect of bile salts that disperse PrPSc–containing membrane phospholipids into small micelles, preventing the aggregation of PrPSc and facilitating its binding to epithelial cells. This observation has significant practical implications because there could be qualitative and/or quantitative differences in the digestive process between individuals and certainly between different species. Such differences, although subtle and apparently trivial, may influence host susceptibility to prion infection from contaminated food.

While purifying PrPSc from CJDH, we noted that the H and L chains of ferritin consistently cosediment with PrPSc. Resistance of the PrPSc–ferritin complex to elution with low concentrations of salt and coimmunoprecipitation with either anti-PrP or anti-ferritin antibodies suggested an association between the two proteins, rather than coincidental sedimentation. Remarkably, both the H and L chains of ferritin resisted PK and DE treatment and were associated with the protease-resistant core of PrPSc. Electron microscopic examination of the 8H4-immunoprecipitated material revealed fibrils decorated with ferritin aggregates. Although other proteins were detected by silver staining of 8H4 and anti-ferritin proteins, indicating the presence of intact PrPSc–ferritin complexes after transcytosis (panels 2–4, arrows). Coimmunostaining of M17 cells for PrP (green) and ferritin (red) shows colocalization of the two proteins, indicating the presence of intact PrPSc–ferritin complexes after transcytosis (panels 2–4, arrows). Coimmunostaining of M17 cells for PrP (green) and streptavidin (red) (panels 6–8) confirms that the PrP signal is derived from biotinylated CJDH-DE in the AP chamber. Scale bar, 10 μm. A, Electron microscopic analysis of the PrPSc–ferritin complex immunoprecipitated with 8H4 shows fibrillar structures and membranes decorated with ferritin aggregates (top inset; arrows). When added to Caco-2 cells, the complex is internalized in relatively large phagosome-like structures surrounded by a single membrane (top; arrowheads). Some of these vesicular structures are extruded out from the BL surface of Caco-2 cells and are seen within the pore of the membrane filter (bottom; arrowhead). (The internalized material in phagosomes shows similar structures as observed in the immunoprecipitated material.) Scale bar: 0.25 μm; inset, 1.5 μm. N, Nucleus; T, tight junction.

Figure 7. sCJD–PrPSc remains associated with ferritin after transcytosis. A, Caco-2 cell monolayers were placed in a 12-well culture dish containing M17 neuroblastoma cells cultured on coverslips in the BL chamber, and biotinylated CJDH-DE was added to the AP chamber (see Fig. 6A). After an overnight incubation, Caco-2 cells on filters and M17 cells on coverslips were processed for immunostaining. Immunoreaction of filters with anti-ZO-1 shows the presence of tight junctions in all monolayers (green; panels 1, 5). Immunostaining of M17 cells in the BL chamber for PrP (green) and ferritin (red) shows colocalization of the two proteins, indicating the presence of intact PrPSc–ferritin complexes after transcytosis (panels 2–4, arrows). Coimmunostaining of M17 cells for PrP (green) and streptavidin (red) (panels 6–8) confirms that the PrP signal is derived from biotinylated CJDH-DE in the AP chamber. Scale bar, 10 μm. B, Electron microscopic analysis of the PrPSc–ferritin complex immunoprecipitated with 8H4 shows fibrillar structures and membranes decorated with ferritin aggregates (top inset; arrows). When added to Caco-2 cells, the complex is internalized in relatively large phagosome-like structures surrounded by a single membrane (top; arrowheads). Some of these vesicular structures are extruded out from the BL surface of Caco-2 cells and are seen within the pore of the membrane filter (bottom; arrowhead). (The internalized material in phagosomes shows similar structures as observed in the immunoprecipitated material.) Scale bar: 0.25 μm; inset, 1.5 μm. N, Nucleus; T, tight junction.

The association of PrPSc and ferritin occurs in vivo or after homogenization of brain tissue is unclear from our data. Nevertheless, this complex is biologically significant because ingested PrPSc in contaminated meat undergoes a process similar to homogenization and DE treatment in the GI tract and is likely presented to the intestinal epithelium in a complex with ferritin. Interestingly, the β-sheet-rich PrP peptide 106–126 mixed with normal or CJ DH homogenate was not transcytosed effectively, indicating that the main determinant of PrPSc transport is not its β-sheet-rich secondary structure. Preincubation of PrP106–126, NH, or CJDH with exogenous purified ferritin did not facilitate the formation of coimmunoprecipitable PrPSc–ferritin complexes, indicating that the association of PrPSc with ferritin is more complex than a mere hydrophobic interaction during the process of homogenization. Regardless of the nature and site of PrPSc–ferritin complex formation, this phenomenon is likely to influence the absorption of ingested PrPSc significantly, especially because ferritin in ingested food is known to undergo active absorption by the human intestinal epithelium (Murray-Kolb et al., 2003; Theil, 2003).
Our results show that the PrPSc-ferritin complex is endocytosed by Caco-2 cells in vesicular structures that fuse to form phagosomes within the cell. Some of these vesicles are transcytosed intact to the BL chamber, much like the reported release of PrPSc-containing exosomes into the extracellular environment by epithelial cells (Fevrier et al., 2004). Sensitivity of the PrPSc–ferritin transport to incubation at low temperature and treatment with brefeldin A and nocodazole suggest the involvement of an active transport process (Klausner et al., 1992). Although Caco-2 cells are known to endocytose ferritin, the mechanistic details of this process remain elusive (Murray-Kolb et al., 2003). Specific receptors for ferritin have been reported on liver cells, erythroblasts, oligodendrocytes, and on various cell lines (Mack et al., 1983; Harrison and Arosio, 1996; Hulet et al., 2000). Our data demonstrating significant inhibition of PrPSc-ferritin uptake in the presence of excess ferritin derived from human liver, spleen, or brain suggests the presence of a ferritin-specific receptor or a transporter on Caco-2 cells. The presence of such a receptor on epithelial cells and the close association of PrPSc and ferritin in digested food incriminate ferritin as a possible transcytotic activity (Heppner et al., 2001; Huang et al., 2002). It is conceivable that endocytosed ferritin is packaged in distinct vesicles that are either targeted to lysosomes or transcytosed to the BL surface. The associated PrPSc in CJDH probably follows both routes, although the majority appears to be transcytosed because very little PrPSc was detected in cell lysates (S. Basu and Singh, unpublished observations). This assumption is supported by the fact that a significant proportion of the PrPSc–ferritin complex remains intact after transcytosis, as evidenced by communostaining of endocytosed aggregates in M17 cells cultured in the BL chamber. PrPSc from untreated NH did not show significant association with ferritin and was not transported to the BL chamber in several experiments. However, ferritin from untreated NH was detected consistently in the BL chamber (Basu and Singh, unpublished observations). Thus, either PrPSc is not endocytosed at all or is degraded within Caco-2 cells. A small amount of PrPSc was detected occasionally independent of associated ferritin. It is unclear whether this fraction is associated with another protein, is transported independently, or results from dissociation of the PrPSc–ferritin complex in an intracellular compartment.

The notion that PrPSc is cotransported with ferritin ignores the key requirements of host susceptibility to prion infection, such as the level of PrPSc expression and the extent of homology between host PrPSc and incoming PrPSc (Prusiner et al., 1990; Weissmann et al., 2002; Thackray et al., 2003). Although in apparent contradiction, our data suggest that the uptake of PrPSc and its subsequent replication are distinct processes. The former is independent of host PrPSc, whereas the latter requires PrPSc as substrate for additional replication. This hypothesis is supported by our data that show no influence of PrPSc overexpression on PrPSc transport across Caco-2 cells and by a recent report demonstrating PrPSc expression below the tight junctions of polarized epithelial cells, making it physically impossible for incoming PrPSc to come in contact with host PrPSc (E. Morel et al., 2004). The cotransport of PrPSc with ferritin raises important questions regarding prion uptake from contaminated food. Although this report uses a homologous experimental setup, ferritin H and L chains are known to share significant homology across species (Harrison and Arosio, 1996) and may facilitate the transport of...
PrP^Sc from distant species across the intestine. Because PrP^Sc is notorious for its sticky nature, ferritin may be only one such carrier protein. The identification and functional role of other proteins associated with DE-treated PrP^Sc is important for fully understanding the mechanism of PrP^Sc uptake from ingested food and preventing a carrier state across species. Heterologous PrP^sc in such carriers may be transported to sites where it may undergo conformational “adaptation” with time (Hill et al., 2000; Race et al., 2001), or in the case of livestock, lie dormant until ingested by a susceptible host. Such apparently “healthy” carriers would disseminate PrP^sc through a variety of means, posing a potential threat to the general population.

References


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