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研究報告の概要

スクレイピー由来の異常プリオン蛋白質（PrP^{Sc}）がヒトの腸管上皮細胞の壁を通過するメカニズムについてはほとんど知られていない。本研究により、弧発型クロイツフェルト・ヤコブ病に感染した脳組織のホモジネートを消化酵素処理したところ、プリオン病の伝播・発症を示唆するプロテイナーゼK耐性PrP^{Sc}のコア部分（27〜30kDa）に類似したC末端断片を産生したことが示された。内因性のPrP^{C}発現量にかかわらず、消化酵素処理により、ヒト腸管上皮細胞のin vitroモデルにおいて、トランスサイトーシスにより小胞構造の形でPrP^{Sc}が取り込まれた。予想外ではあったが、PrP^{Sc}は酵素処理したPrP^{Sc}ー蛋白質複合体の主な成分であるフェリチンと共に運ばれる。PrP^{Sc}ーフェリチンの輸送は低温、ブレフェルディンAやノコダゾールの処理に感受性であり、遊離したフェリチンが過剰になると抑制される。このことは、受容体あるいはトランスポーター媒介の輸送経路の存在を示唆する。フェリチンは種の間でよく保存されているため、遠く離れた種のPrP^{Sc}が腸管から吸収されるのを促進し、ヒトにおける輸送体となる可能性があることを示唆する。

報告企業の意見	今後の対応
フェリチンが種の間でよく保存されているため、遠く離れた種のPrP^{Sc}が腸管から吸収されるのを促進し、ヒトにおける輸送体となる可能性があることを示した新たな知見である。	今後も引き続き、プリオン病に関する新たな知見について情報収集に努める。

使用上の注意記載状況・その他参考事項等

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血液を介するウイルス、細菌、原虫等の感染
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19

Neurobiology of Disease

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, Xiu 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 (PrP^{Sc}), 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 PrP^{Sc} core of 27–30 kDa implicated in prion disease transmission and pathogenesis. Notably, DE treatment results in a PrP^{Sc}–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^{C} expression. Unexpectedly, PrP^{Sc} is cotransported with ferritin, a prominent component of the DE-treated PrP^{Sc}–protein complex. The transport of PrP^{Sc}–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 PrP^{Sc}-associated proteins, in particular ferritin, may facilitate PrP^{Sc} 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 (PrP^{Sc}) 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 PrP^{Sc}-contaminated food products continue to increase, it has become increasingly critical to understand the mechanism by which PrP^{Sc}, 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 PrP^{Sc} 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 PrP^{Sc} 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 PrP^{Sc} 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 PrP^{Sc} uptake from contaminated food by the intestinal epithelial cells, we investigated the transport of human PrP^{Sc} from sporadic CJD brain tissue (sCJD–PrP^{Sc}) 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-

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*R.S.M. and S.B. contributed equally to this work.

Correspondence should be addressed to Dr. Neena Singh, Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106. E-mail: neena.singh@case.edu.

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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 PrP^{Sc} (PrP27–30) implicated in the transmission and pathogenesis of prion disorders (Prusiner, 1998). Unexpectedly, both PK and DE treatments generate a PrP^{Sc}–protein complex that includes ferritin as a major component, and the PrP^{Sc}–ferritin complex is cotransported across Caco-2 cells in vesicular structures. The transport of PrP^{Sc}–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 PrP^{Sc} 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 144–152; 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). RITC- 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% CO_2 atmosphere and passaged weekly. For preparing monolayers, cells from a confluent flask were resuspended in DMEM at a concentration of 2×10^6 cells/ml and added to the apical (AP) chamber of polycarbonate filters [Transwell; 12 or 24 mm diameter (1 and 4.7 cm^2, respectively); 3 μm pore size; Costar, Cambridge, MA]. The filters were placed in a 12- or 6-well culture dish containing 0.6 or 1.2 ml of DMEM, respectively. The medium was replaced every day until confluent monolayers with tight junctions developed (10–14 d). The integrity of tight junctions was monitored by measuring transepithelial electrical resistance (TEER) across the monolayer with a millicell-ERS instrument (Millipore, Bedford, MA) and by measuring the transfer of ^{3}H-inulin from the AP to the basolateral (BL) chamber. Monolayers exhibiting a TEER of >400 Ω/cm^2 and a ^{3}H-inulin transport of <0.01%/cm^2/min after 1 hr of incubation at 37°C were used for transport studies. For some studies, M17 cells cultured on polylysine-coated glass coverslips were placed in the BL chamber for the duration of the experiment.

Transfection of Caco-2 cells. The coding sequence of human PrP was subcloned into the eukaryotic expression vector cep4β using the *Not*I and *Bam*HI restriction sites as described previously (Petersen et al., 1996) and transfected into Caco-2 cells with LipofectAMINE according to the manufacturer's (Invitrogen, Grand Island, NY) specifications. Transfected cells were selected and maintained in selective medium (500 μg/ml hygromycin) at 37°C in a humidified atmosphere supplemented with 10% CO_2.

Preparation of DE- and PK-treated brain homogenates and immunoprecipitation. For sample preparation, 0.1 gm of brain tissue from the frontal cortex was sonicated in 1 ml of PBS to obtain a 10% homogenate. Treatment with DEs was performed as described by Glahn et al. (1998). In short, 0.5 ml of 10% normal homogenate (NH) or sCJD homogenate (CJDH) in PBS was treated with 200 U of salivary amylase at 37°C for 15 min. The pH of the solution was adjusted to 2.0 with 5.0 M HCl, and 0.05 ml of pepsin (4095 U) was added. After additional rocking at 37°C for 1 hr, the pH was raised to 6.0 with 1 M sodium bicarbonate, and 0.2 ml of pancreatin–bile extract was added (0.00185 gm of pancreatin and 0.011 gm of bile extract/ml of 0.1 M $NaHCO_3$). The pH was raised to 7.4 with 6N sodium hydroxide, and 0.0084 ml each of 2 M NaCl and KCl solutions was added. The mixture was again rocked at 37°C for 2 hr. At the end of the digestion, the added enzymes were inactivated with 4 mM PMSF and a mixture of protease inhibitors containing 10 μg/ml each of leupeptin, antipain, and pepstatin, and the digest was stored at −70°C for additional use. The same mixture of protease inhibitors was used throughout this study.

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 PrP^{Sc} from sCJD brain homogenate. For the isolation of purified PrP^{Sc}, 0.3 gm 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 centricon 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 ultracentrifuged 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 PrP^{Sc}-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 P6 obtained from both sCJD and normal brain tissue was resuspended 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 PrP^{Sc} 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, electroblotted to a polyvinylidene difluoride (PVDF) membrane, and probed with specific antibodies.

Quantitative analysis was performed by measuring the total raw density of PrP^{Sc} 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^{Pellet} or $CJDH^{Pellet}$ suspension mixed in 84 μl of PBS containing 1% BSA or 0, 0.5, 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 5105 confocal microscope (Zeiss, Oberkochen, Germany).

Electron microscopy (transmission electron microscopy). Caco-2 cells on filter inserts were exposed to 8H4-immunoprecipitated CJDH–PrP^{Sc} 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 inserts 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 in 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–PrP^{Sc} is partially proteolyzed by DEs

PrP^{Sc} 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 PrP^{C} and PrP^{Sc} are not known. To address this question, samples of NH and CJDH were subjected to sequential treatment with DE to simulate the *in vivo* conditions. Beginning with amylase at pH 7.4, the homogenates were sequentially treated with pepsin at pH 2.0, followed by pancreatin and bile extract at pH 6.0.

The effect of DE on PrP^{Sc} was compared 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 unglycosylated forms of PrP^{C} migrating at 35–37, 28–30, and 27 kDa respectively (Fig. 1*A*, lane 1). Similar glycoforms are detected in mock-treated CJDH, although the ratio of the three glycoforms is altered (Fig. 1*A*, lane 4). Treatment with PK or DE results in complete proteolysis of PrP^{C} in NH (Fig. 1*A*, lanes 2, 3), whereas CJDH samples show faster migrating forms of 27–30 and 19 kDa, consistent with the migration of infectious

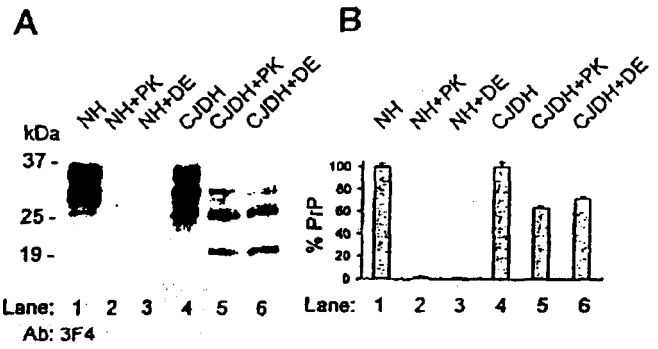

Figure 1. Human sCJD–PrP^{Sc} is partially proteolyzed by DEs. *A*, Immunoblotting of NH and CJDH with 3F4 shows the three glycoforms of PrP migrating at 35–37, 28–30, and 27 kDa (lanes 1, 4). Treatment of NH with PK or DE results in almost complete digestion of PrP^{C} (lanes 2, 3), whereas similar treatment of CJDH results in N-terminally truncated PrP^{Sc} forms migrating at 19–30 kDa (lanes 5, 6). *B*, Densitometric analysis indicates hydrolysis of 97–98% of PrP^{C} in NH by PK or DE, whereas PrP^{Sc} in CJDH shows limited resistance to PK (68%) and DE (75%).

and pathogenic PK-resistant PrP^{Sc} (Fig. 1*A*, 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 PrP^{Sc} by the two procedures. Additional confirmation of DE-mediated cleavage of PrP^{Sc} 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 PrP^{Sc} 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. 1*B*). Thus, by the time ingested PrP^{Sc}-contaminated meat reaches the intestine, almost all of PrP^{C} is proteolyzed, and PrP^{Sc} is converted to the protease-resistant C-terminal core of 27–30 kDa.

DE-treated sCJD–PrP^{Sc} is transported across Caco-2 epithelial cells

The transport of PrP^{C} and PrP^{Sc} 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 ^{3}H-inulin transport from the AP chamber to the BL chamber of <0.01%/cm²/min were used. Transport of ^{3}H-inulin was compared before and after each treatment to rule out any toxic effects of the homogenate or PrP^{Sc} during the experiment. Each sample was tested in duplicate, and each experiment was repeated at least five times.

In a typical experiment, 20 μl of NH or CJDH that had been mock treated, PK treated, DE treated, or DE treated and mixed with the PrP peptide 106–126 (10 μM) was added to the AP 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 is recovered from the AP medium, indicating negligible transport to the BL chamber (Fig. 2*A*, lanes 1–3). As expected, NH-PK and NH-DE samples show barely detectable PrP signal in the AP or

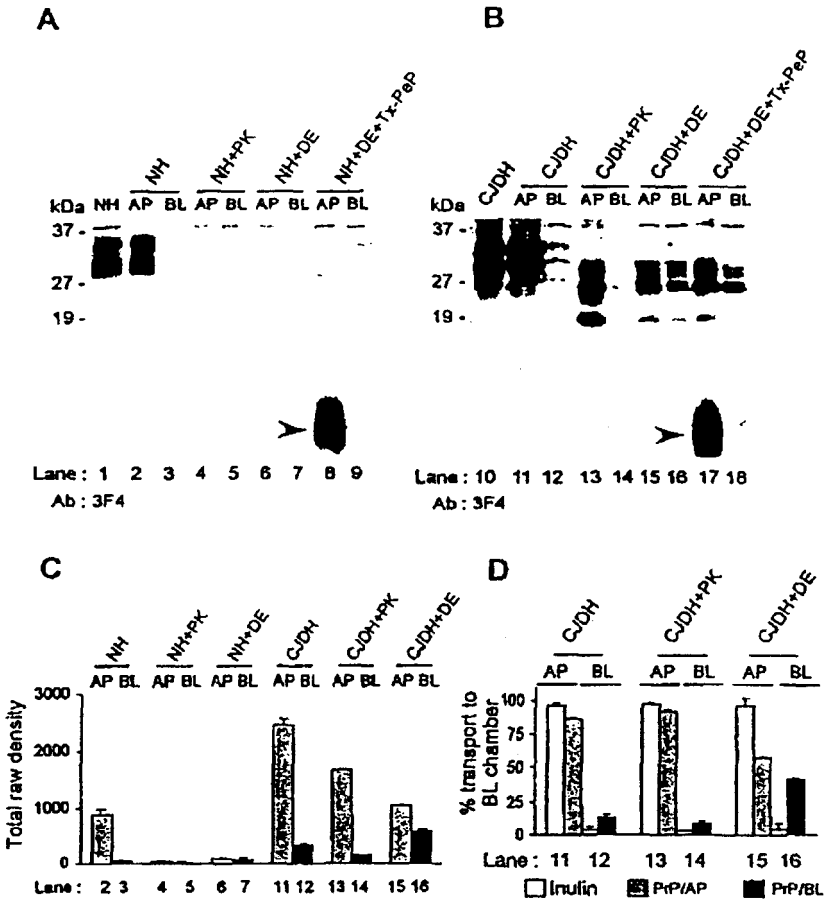

Figure 2. DE-treated sCJD–PrP^{Sc} is transported across Caco-2 epithelial cells. An aliquot of mock-treated, PK-treated, or DE-treated NH or CJDH was added to the AP chamber of Caco-2 monolayers, and PrP transported to the BL chamber was estimated after an overnight incubation. *A*, Immunoblotting of proteins from the AP and BL media with 3F4 shows no detectable PrP signal in the BL chamber of cells exposed to mock-treated (lanes 2, 3), PK-treated (lanes 4, 5), or DE-treated (lanes 6–9) NH. *B*, In contrast, a significant PrP signal is detected in the BL chamber of cells exposed to mock-treated (lanes 11, 12), PK-treated (lanes 13, 14), or DE-treated (lanes 15–18) CJDH. PrP peptide 106–126 (Tx-pep) is not transported to the BL chamber when mixed with either DE-treated NH (*A*; lanes 8, 9, arrowhead) or DE-treated CJDH (*B*; lanes 17, 18, arrowhead). Lanes 1 and 10 show PrP reactivity in the starting NH and CJDH samples, respectively. *C*, Measurement of total raw density of PrP signal indicates minimal transport of PrP from mock-treated NH. In PK-treated and DE-treated NH samples, as expected, minimal PrP signal is detected. In contrast, 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*). *D*, 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 ^{3}H-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. $p < 0.01$.

BL samples (Fig. 2*A*, lanes 4–7). However, PrP106–126 (toxic peptide) is easily detected in the AP medium of NH-DE plus toxic peptide sample, ruling out experimental error in the detection of any leftover PrP (Fig. 2*A*, lane 8, arrowhead). In contrast, PrP in CJDH and protease-resistant PrP^{Sc} in PK- and DE-treated CJDH samples are transported to the BL chamber (Fig. 2*B*, lanes 10–16). Surprisingly, PrP^{Sc} in DE-treated CJDH is transported more efficiently than from the PK-treated sample (Fig. 2*B*, lanes 13–18). The PrP106–126 peptide mixed with CJDH-DE is not transported, although a bold PrP^{Sc} signal is detected in the BL chamber of CJDH-DE plus toxic peptide sample (Fig. 2*B*, 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 Figure 2, *C* 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. 2*C*, 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. 2*C*, lanes 4–7). In contrast, a significant proportion of PrP^{Sc} from the CJDH samples is transported from the AP to the BL chambers (Fig. 2*C*, lanes 11–16), representing a transport of 14, 8.5, and 42% of PrP^{Sc} from mock-treated, PK-treated, and DE-treated CJDH, respectively (Fig. 2*D*, lanes 11–16). The transport of ^{3}H-inulin across the same monolayer is $<0.01\%/cm^2/min$ in 1 hr at 37°C (Fig. 2*D*), confirming the integrity of tight junctions under these experimental conditions.

Overexpression of PrP^C does not alter the transport of sCJD–PrP^{Sc} across Caco-2 cells

To evaluate whether the level of PrP^C expression on Caco-2 cells influences PrP^{Sc} uptake or transport, Caco-2 cells were transfected with a plasmid encoding human PrP^C, and the percentage of increase in PrP^C 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 PrP^C expression by Caco-2^{PrP} cells compared with nontransfected Caco-2 cells and 1.25 times that of M17 neuroblastoma cells (Fig. 3*A*, lanes 1–3, *B*).

The influence of increased PrP^C expression on PrP^{Sc} transport was estimated by isolating a crude fraction of PrP^{Sc} from PK-treated CJDH to avoid the influence of membrane in mediating PrP^{Sc} 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. After an overnight incubation, AP and BL media were analyzed by immunoblotting and densitometric analysis as above. No significant difference is observed in the amount of PrP^{Sc} transported across Caco-2^{PrP} compared with nontransfected Caco-2 cells (Fig. 3*C*, lanes 1–4, *D*). These results were further confirmed by comparing the transport of PrP^{Sc} across HT-29 cells, another human intestinal epithelial cell line that expresses twofold more PrP^C than Caco-2. No significant difference was observed in the rate or quantity of PrP^{Sc} transported across Caco-2 and HT-29 cells (data not shown), suggesting that the host PrP^C expression level does not influence the internalization of PrP^{Sc} by epithelial cells.

Protease-resistant human sCJD–PrP^{Sc} is associated with ferritin

We next attempted to purify PrP^{Sc} 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^{Sc} 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. 4*A*, lane 1). After the first round of ultracentrifugation, ~40% of PrP^{Sc} fractionates in the supernatant fraction (S2), and ~60% is detected in the pellet (P2) (Fig. 4*A*, lanes 2, 3). In subsequent rounds, all of the PrP^{Sc} is detected in the pellet fractions (P3–P6) (Fig. 4*A*, lanes 5, 7, 9, 11).

To assess the purity of PrP^{Sc} 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^{Sc} are identified as in Figure 4*A* (Fig. 4*B*, lane 1). In addition, a prominent band migrating at 20 kDa is seen (Fig. 4*B*, lane 1). After deglycosylation, PrP^{Sc} glycoforms collapse to 19 kDa (Fig. 4*B*, lane 2, arrowhead), whereas the 20 kDa band remains unchanged (Fig. 4*B*, lane 2, arrow). The band marked with an asterisk represents the added PNGase (Fig. 4*B*, 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 reprobing the membrane in Figure 4*A* with anti-ferritin antibody. Strong immunoreaction is detected with the 20 kDa band, confirming its identity as ferritin (Fig. 4*C*, lanes 1–11). It is remarkable that ferritin resists PK treatment and persistently pellets with PrP^{Sc}.

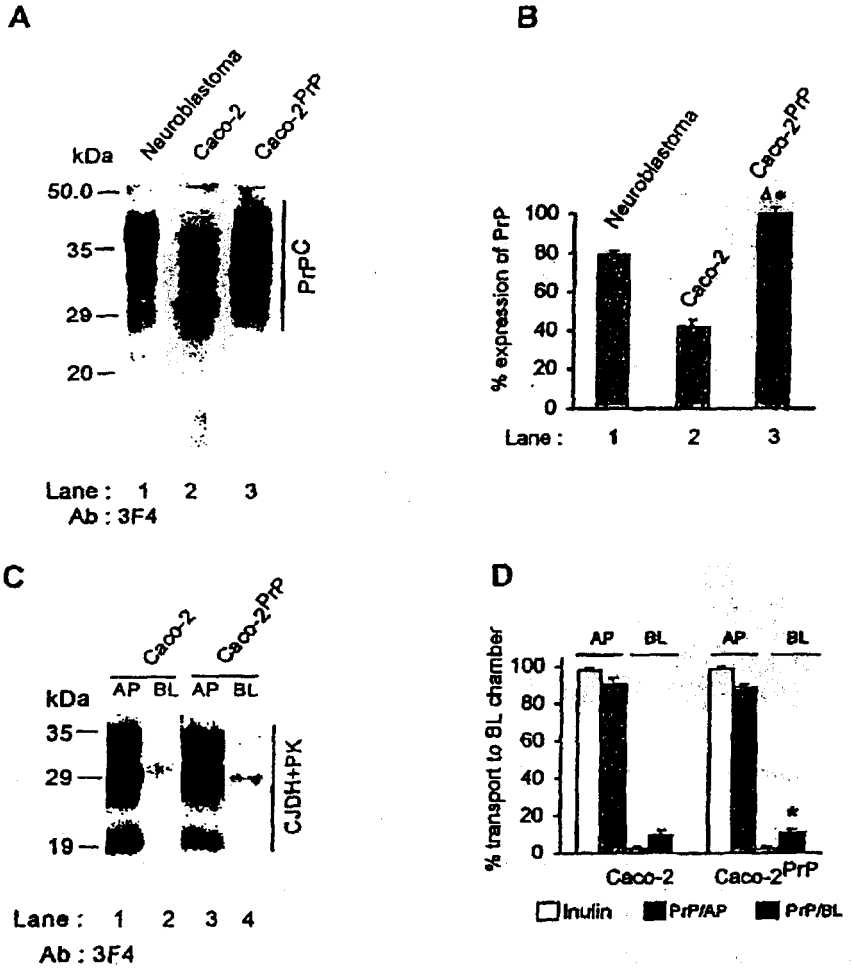

Figure 3. Overexpression of PrP^{C} does not influence the transport of sCJD–PrP^{Sc} across Caco-2 monolayers. Lysates of M17 neuroblastoma cells, Caco-2 cells, and transfected Caco-2^{PrP} cells were immunoblotted with 3F4 to assess the level of PrP expression. *A*, Transfected Caco-2^{PrP} expresses significantly more PrP compared with M17 and nontransfected Caco-2 cells (lane 3 vs lanes 1, 2). *B*, Quantitative estimation shows 2.5- and 1.5-fold PrP expression in Caco-2^{PrP} cells compared with nontransfected Caco-2 and M17 cells, respectively (lane 3 vs lanes 1 ($^{*}p < 0.05$; $n = 3$) and lane 3 vs lane 2 ($^{\Delta}p < 0.01$; $n = 3$)). *C*, Transport of PK-resistant PrP^{Sc} across Caco-2 and Caco-2^{PrP} cell monolayers was measured as in Figure 2. There is no significant difference in the amount of PrP^{Sc} transported to the BL chamber of Caco-2^{PrP} cells compared with nontransfected Caco-2 cells (lanes 3, 4 vs lanes 1, 2). *D*, Quantitative estimation shows transport of 10 and 11.2% of PrP^{Sc} to the BL chamber of Caco-2 and Caco-2^{PrP} cell monolayers, respectively. Each bar represents the mean ± SD of three experiments. $^{*}p < 0.05$.

The above results argue that either PrP^{Sc} 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. 4*D*, bottom, lanes 1–6). Almost all of the PrP^{Sc} is retained in the column and is barely detected in the eluate (Fig. 4*D*, top, lanes 1–6).

Thus, PrP^{Sc} 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^{Sc}–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^{Sc} 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^{Sc} is similarly associated with ferritin, mock-treated and DE-treated NH and CJDH were clarified by centrifugation at 3000 × *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. 5*A*, lanes 1, 2). In contrast, surprisingly large