Bovine Prion Is Endocytosed by Human Enterocytes via the 37 kDa/67 kDa Laminin Receptor

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Some forms of transmissible spongiform encephalopathies result from oral infection. We have thus analyzed the early mechanisms that could account for an uptake of infectious prion particles by enterocytes, the major cell population of the intestinal epithelium. Human Caco-2/TC7 enterocytes cultured on micro-porous filters were incubated with different prion strains and contaminated brain homogenates in the apical compartment. Internalization of infectious particles was analyzed by Western blotting and immunofluorescence. We observed internalization by enterocytes of prion particles from bovine spongiform encephalopathy brain homogenates but not from mouse-adapted scrapie-strain brain homogenates or purified bovine spongiform encephalopathy scrapie-associated fibrils. Bovine prion particles were internalized via endocytosis within minutes of infection and were associated with subapical vesicular structures related to early endosomes. The endocytosis of the infectious bovine PrP^{Sc} was reduced by preincubating the cells with an anti-LRP/LR blocking antibody, identifying the 37 kDa/67 kDa laminin receptor (LRP/LR), which is apically expressed in Caco-2/TC7 cells, as the receptor for the infectious prion protein. Altogether, our results underscore a potential role of enterocytes in the absorption of bovine prions during oral infection through specific LRP/LR-dependent endocytosis. (*Am J Pathol* 2005, 167:1033–1042)

Prions (PrP^{Sc}, scrapie prion protein) are infectious proteins that correspond to the pathological isoform of the cellular prion protein PrP^C. They are thought to be the causative agents of transmissible spongiform encephalopathies, which affect humans (Kuru, fatal familial insomnia, or Creutzfeldt-Jakob disease), and animals [scrapie, bovine spongiform encephalopathy (BSE), or chronic wasting disease].¹

The oral transmission of infectious prion particles from cattle to humans results in the development of the variant form of Creutzfeldt-Jakob disease.^{2,3} The accumulation of bovine PrP^{Sc} in Peyer's patches after oral infection in animal models^{4–7} clearly implies that prions cross the intestinal epithelial barrier. However, up to now, no study has concerned the early mechanisms leading to the internalization of prion particles in human intestinal cells after the oral ingestion of bovine prion-infected tissues. It has been proposed that M cells could manage such an uptake,⁸ considering that these cells are present in the covering epithelium of Peyer's patches and display a high phagocytotic activity. However, previous results obtained in neonatal mice⁹ and in primates¹⁰ have also shown the presence of PrP^{Sc} in enterocytes after oral exposure to prion strains. Enterocytes represent the major cell population of the intestinal epithelium,¹¹ even at the level of Peyer's patches,¹² and are known to actively participate in endocytosis of nutrients, macromolecules, or pathogens through their polarized traffic equipment.¹³ Human enterocytes have been shown to express the 37 kDa/67 kDa laminin receptor in their apical brush border.^{6,14} In nerve cells, this protein was demonstrated to be a receptor for prion proteins and to play a role in their endocytosis and recycling.^{15–21} Moreover, we have recently shown that human enterocytes and the enterocyte-like Caco-2/TC7 cells endogenously express PrP^C.²² All

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In memoriam of Dominique Dormont.

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together, these data led us to hypothesize that enterocytes might play an important role for the uptake of infectious prion particles and might represent a first site for PrP^C transconformation inside the intestinal epithelium during oral infection.

Using as a model system the human Caco-2/TC7 cells, which display most of the morphological and functional characteristics of normal human enterocytes,^{23,24} we demonstrate the specificity of bovine prion uptake in human enterocytes. Bovine prion is rapidly endocytosed through the 37 kDa/67 kDa laminin receptor and trafficked toward early endosomes structures and most probably to lysosomes.

Materials and Methods

Reagents and Antibodies

All chemicals were purchased from Sigma (St. Quentin Fallavier, France), except when indicated. Mouse monoclonal 8G8, SAF32, SAF54, SAF83-HRP, 12F10 anti-prion antibodies were from SPI-BIO (Massy, France). Mouse monoclonal SAF60 and Pri-308 anti-prion antibodies were from J.G.'s laboratory. Rabbit polyclonal anti-LAMP2 (lysosomal-associated membrane protein 2), anti-mouse and anti-rabbit horseradish peroxidase antibodies were from Santa Cruz (TEBU, Le Perray en Yvelines, France). Rabbit polyclonal anti-EEA1 (early endosome antigen 1) and mouse monoclonal anti- α 6 integrin antibody were purchased from Alexis Biochemicals (COGER, Paris, France). Rabbit polyclonal anti-human ZO1 and rat monoclonal (ECCD2) anti-E-cadherin antibodies were from Zymed Laboratories (Clinisciences, Montrouge, France). The rabbit polyclonal anti-LRP/LR W3 antibody is from S.W.'s laboratory. F-actin was labeled with phalloidin-fluorescein isothiocyanate (Sigma). Secondary donkey Cy2- and Cy3-labeled antibodies were from Jackson ImmunoResearch.

Prion Strains and Brain Homogenate Preparation

BSE-infected bovine brain samples were from J.G.'s laboratory. Scrapie-infected mouse brain samples were from C57/BL6 mice at the terminal stage of the disease after intracerebral inoculation of 100 $\times$ LD50 of C506M3 strain (obtained from P. Brown, National Institutes of Health, Bethesda, MD). Noninfectious bovine or mouse brain samples were from healthy cow or C57/BL6 mice. Infectious or noninfectious brain samples were homogenized at 20% (wt/vol) in a 5% glucose (wt/vol) sterile solution and diluted for immediate use in cultures without proteinase K treatment. To prepare BSE or scrapie-associated fibrils, infectious brain homogenates (100 to 200 μ l) were treated with proteinase K (30 μ g/ml final concentration; Eurobio, Les Ulis, France), as previously reported.²⁵ An aliquot of BSE fibrils was resuspended in a 5% glucose solution for immediate use in cultures. BSE and scrapie fibrils were denatured in a 4215 buffer (4% sodium dodecyl sulfate, 2% β -mercaptoethanol, 157 mmol/L Tris-

HCl, pH 6.8, 5% sucrose, and bromophenol blue) to be used as controls for polyacrylamide gel electrophoresis analysis and immunoblotting.

Cell Incubation

Caco-2/TC7 cells were cultured on 3- μ m pore size microporous PET filters (Falcon, BD Biosciences, Le Pont de Claix, France), as previously described.²⁶ For prion infection, 20 μ l of 10% brain homogenates (mouse scrapie or bovine BSE) or of 10% BSE-SAFs, were applied in the apical medium. The same volumes and concentration of murine or bovine noninfectious brain homogenates were used for control conditions. Cytotoxicity was checked by the measure of MTT activity in the cells (MTT assay kit, Sigma) and of lactate dehydrogenase activity in the apical medium (lactate dehydrogenase assay kit, Sigma). The *trans*-epithelial electric resistance was measured with Millicell-ERS apparatus (Millipore, St. Quentin en Yvelines, France).

Figure 1. Specific internalization of bovine prions in human enterocytes. Detection of proteinase K-resistant prion protein by Western blot analysis of total cell lysates from Caco-2/TC7 cells incubated for 10 minutes, 3 hours, or 24 hours with apically supplied scrapie prion strain-infected mouse brain homogenates (A), or bovine spongiform encephalopathy (BSE)-infected brain homogenates (B), or fibrils purified from BSE brain homogenates (BSE-SAFs) (C). SAF83 and SAF60 antibodies were, respectively, used for murine scrapie prion strain and for bovine prion detection. Samples were normalized to equal protein concentrations. Murine or bovine NIH, PK-treated Caco-2/TC7 cells lysates after 24 hours of incubation with apically supplied NIHs of bovine or mouse brains; ctrl-, PK-treated normal cell lysates; ctrl+, PK-treated fibrils purified from BSE or scrapie-infected brain homogenates used as positive controls for each prion species.

Figure 2. Integrity of Caco-2/TC7 cells is preserved during infection with BSE brain homogenates. *Trans*-epithelial electrical resistance (Ω / cm^2) (**A**), apical release of lactate dehydrogenase (**B**), and MTT activity (**C**) were measured in Caco-2/TC7 cells after 24 hours of incubation without (ctrl) or with NIH or BSE homogenates. **D** and **E**: Immunofluorescence analysis of cell morphology. The localization and distribution of epithelial markers such as tight junction-associated protein zonula occludens 1 (ZO1), adherens junction component E-cadherin, actin cytoskeleton (F-actin), brush border-associated sucrase-isomaltase (SI), and of the endogenous cellular prion protein (PrP^c) have been compared between control conditions (ctrl) and after 24 hours of incubation with BSE brain homogenates. ZO1, E-cadherin, and PrP^c are visualized in planes crossing lateral membranes, SI is restricted to the brush-border domain (BBD) in XY and XZ representations. F-actin is visualized in the BBD of the apical plane in XY panels and in BBD and lateral junctions in XZ representation. **Dashed gray lines** represent the basal pole of the cells in the XZ projection. Scale bars, 20 μm .

Cell Lysates, Western Blot, and Dot-Blot Analyses

After 1, 5, 15 minutes, 3 hours, or 24 hours of infection, the apical medium was removed and cells were rinsed with fresh medium without control or infectious brain homogenates or fibrils, then extensively washed in cold phosphate-buffered saline (PBS). Cells were scrapped in lysis buffer (50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100). After normalization of the protein content (Optima Interchim kit, Interchim, Montulçon, France), resulting cell lysates were digested (40 minutes, 37°C) with PK at 20 μ g/ml, a concentration that was found sufficient to totally digest PrP^{Sc}. Cell lysates were then centrifuged (90 minutes, 20,000 $\times$ g) and the pellet was suspended in denaturing buffer and heated (5 minutes, 100°C). For Western blot analyses, samples were run in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with sucrose (7.5%) and electrotransferred overnight on nitrocellulose membranes (Amersham, Orsay, France). Membranes were blocked 2 hours with 5% nonfat dried milk in PBS-Tween 20 (0.1%) (PBST), incubated (1 hour, room temperature) with specific anti-PrP or anti-LRP/LR antibodies and with horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulins (Santa Cruz) in PBST. Bound antibodies were detected by chemiluminescent method (ECL+, Amersham). For dot-blot analyses, PrP^{Sc} was extracted from BSE-infected brain homogenates (100 to 200 μ l)²⁵ and was not subjected to PK digestion. Protein pellets were resuspended in H₂O and incubated with 5% glucose solution (ratio, 1:3) at different pH (pH 5, 5.5, 6, and 7). Five μ l of each sample were directly blotted on a nitrocellulose membrane and analyzed by Western blot.

Immunofluorescence Analyses

Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Primary and secondary antibodies were diluted in blocking buffer (PBS, bovine serum albumin 0.1%) and incubated 1 hour at room temperature. Immunofluorescence backgrounds were estimated by the signal obtained in cells incubated with noninfectious brain homogenates or by the fluorescence of infected cells when secondary antibodies were applied alone. For immunofluorescence analyses of bovine brain homogenates, a drop of 20% brain homogenate was spotted on a coverslip, washed with PBS after drying, fixed with Celfix reagent (BD Biosciences), and incubated overnight at 4°C in PBS. Pseudo-permeabilization was performed in 0.1% Triton X-100. First and secondary antibodies were diluted in blocking buffer (PBS, bovine serum albumin 0.1%) and incubated at room temperature. Immunofluorescence was examined by classical (Axiophot Zeiss, Zeiss, Le Pecq, France) or confocal (Zeiss LSM 510) fluorescence microscopy.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test.

Results

Bovine BSE Prion Is Specifically Internalized in Human Caco-2/TC7 Enterocytes

We first analyzed the presence of PrP^{Sc} in polarized Caco-2/TC7 enterocytes as a function of incubation time under an apical supply of prion-contaminated brain homogenates from different sources. No PrP^{Sc} signal was detected in PK-treated lysates of cells incubated with noninfectious control brain homogenates (NIH lanes) or incubated with brain homogenates infected with murine C506M3 scrapie strain (Figure 1A) that was reported to be taken up by murine intestinal cells.⁵ Conversely, we observed an accumulation of proteinase K-resistant PrP in cells incubated with crude BSE brain homogenates (Figure 1B). Although barely detectable after 10 minutes, bovine PrP^{Sc} was easily detected in cell lysates after 3 or 24 hours of incubation. Interestingly, no signal was detected after incubation with bovine PrP^{Sc} purified as fibrils (BSE-SAFs) (Figure 1C). These results demonstrate that Caco-2/TC7 enterocytes can internalize bovine prion from the apical medium and that these particles can accumulate in the cells. Conversely, murine prion or pu-

Figure 3. 8G8 monoclonal anti-prion antibody discriminates between bovine PrP^{Sc} and endogenous PrP^c of Caco-2/TC7 cells in immunofluorescence analysis. **A:** Specificity of different antibodies in the detection of BSE-associated PrP^{Sc} isoform in Western blot and of endogenous PrP^c in Caco-2/TC7 cells and of BSE brain homogenates in immunofluorescence analyses. **B:** Detection by immunofluorescence of bovine prion with 8G8 antibody in BSE brain homogenates fixed on glass lamellae (**top**). The specificity of the signal obtained with 8G8 antibody was confirmed by staining of the same BSE brain homogenates with IgG2a immunoglobulins followed by fluorescent secondary antibody (**bottom**). **C:** Detection by immunofluorescence and analysis of the distribution of PrP^c in control Caco-2/TC7 cells with 8G8 antibody (**top**) or with 12F10 antibody (**bottom**). Scale bars, 20 μ m. Original magnifications, $\times 10$ (**B**).

rified BSE fibrils cannot enter enterocytes. No human PrP^{Sc} could be detected by Western blot analysis of infected cells using the Pri-308 antibody, which specifically recognizes the human isoform (data not shown),²⁷ whereas bovine PrP^{Sc} was detected using the SAF60 antibody (Figure 1B), indicating that bovine PrP^{Sc}/human PrP^C transconformation had not occurred at early stages of infection.

Cell Morphology and Endogenous PrP^C Localization Are Not Affected by Incubation of Caco-2/TC7 Enterocytes with Bovine Prion

We next analyzed potential deleterious effects of bovine prion infection on the integrity and morphology of Caco-2/TC7 cell monolayer. *Trans*-epithelial electric resistance did not vary after 24 hours (Figure 2A) or 48 hours (not shown) of incubation. Measurements of MTT activities and of the lactate dehydrogenase released in the apical medium indicate that bovine prion did not induce any cytotoxicity in Caco-2/TC7 cells incubated with infectious brain homogenates, as compared to untreated control

cells (control) and to cells incubated with noninfectious homogenates (NIHs) (Figure 2, B and C). Cell morphology and polarization were further studied by immunofluorescence analysis of the expression and of the subcellular distribution of epithelial markers. Zonula occludens 1 (ZO1), the tight junction-associated protein, and E-cadherin, the major component of adherens junctions, were located at lateral membranes in infectious as well as in control conditions (Figure 2D). Prion incubation did not induce any change in the morphological characteristics of these enterocyte-like cells, as assessed by the apical brush-border microvilli-associated F-actin, by the lateral actin belt and by the presence of the enterocyte-specific sucrase-isomaltase marker in the brush-border domain (Figure 2E; F-actin and SI staining, XZ projection). Finally, we checked the subcellular localization of the normal cellular isoform of prion protein PrP^C, since we recently showed that it is expressed at the lateral cell-cell contacts of enterocytes and that this localization is impaired by perturbation of cell polarity.²² Bovine prion entry in Caco-2/TC7 cells did not induce any modification in PrP^C distribution (Figure 2D, bottom).

Figure 4. Endocytosis and fate of prion-containing vesicles after incubation of Caco-2/TC7 cells with BSE brain homogenates. **A:** Staining of prions with 8G8 antibody (red channel) and of F-actin (green channel) after 5 minutes of incubation of Caco-2/TC7 cells with BSE brain homogenates in the apical medium. In confocal XZ representation, **dashed gray line** represents the basal pole of the cells. Note the presence of vesicles containing endocytosed bovine prion in the subapical compartment of Caco-2/TC7 cells (at the base of the brush-border-associated F-actin, **open arrows**). **Bottom right:** Absence of signal with 8G8 antibody after 5 minutes of incubation of Caco-2/TC7 cells with noninfectious bovine brain (NIH) homogenates. **B:** Immunofluorescence analysis of prion-containing vesicles (red channel, 8G8) and early endosome-associated antigen-1 protein (green channel, EEA1) in the subapical compartment of Caco-2/TC7 cells after 5 minutes of incubation with BSE brain homogenates in the apical medium. **Arrowheads** show bovine prion and EEA1 co-localization. **C:** Immunofluorescence analysis of prion-containing vesicles stained with 8G8 antibody after 5 minutes (**top**) or 24 hours (24 hours) of incubation with BSE brain homogenates in the apical medium. **D:** Western blot analysis of bovine PrP^{Sc} in PK-treated lysates of Caco-2/TC7 cells after 5 minutes and 24 hours of incubation with BSE brain homogenates (BSE) or 24 hours of incubation with noninfectious brain homogenates (NIH) in the apical medium. **E:** Dot-blot analysis of native bovine PrP^{Sc} with 8G8 or SAF32 antibodies as a function of pH. Prion-infected bovine brain homogenates were prepared in buffers at the indicated pH, without PK treatment. A representative dot-blot is shown in the **top** panels. Graphs represent results from three independent experiments (**P < 0.01 as compared with values obtained at pH 7). Note that prion was easily detected at pH 5.5 by SAF32 antibody, which unfortunately does not discriminate between human PrP^C and BSE PrP^{Sc} in immunofluorescence analysis. **F:** Immunofluorescence analysis of prion (red channel, 8G8)- and LAMP2 (green channel, LAMP2)-containing vesicles in the subapical compartment of Caco-2/TC7 cells after 15 minutes of incubation with BSE brain homogenates in the apical medium. **Open arrowheads** show lysosome-associated LAMP2 and **white arrowheads** show prion-containing vesicles. Scale bars: 10 μ m (**A**, **B**, **F**); 20 μ m [**A** (merge xz and prion 8G8), **C**].

Endocytosis and Fate of Prion-Containing Vesicles after Incubation of Caco-2/TC7 Cells with BSE Brain Homogenates

To characterize the early steps of prion uptake by immunofluorescence, we used the 8G8²⁸ antibody. Indeed, among six antibodies that were screened (Figure 3A), 8G8 was the only one to recognize bovine prion PrP^{Sc} in Western blotting (data not shown) and by immunofluorescence microscopy on BSE brain homogenates (Figure 3B), without any detection of the endogenous human PrP^C in Caco-2/TC7 cells (Figure 3C, top), although PrP^C expression was confirmed by 12F10 monoclonal antibody staining (Figure 3C, bottom). In this experiment, SAF60 monoclonal antibody was used as a positive control for bovine PrP^{Sc} detection in Western blot analysis (T.A. and J.G., unpublished results). Finally, the specificity of the detection of bovine PrP^{Sc} with 8G8 antibody in immunofluorescence analyses was further confirmed in Caco-2/TC7 cells that were incubated with bovine noninfectious brain homogenates (NIHs) (Figure 4A, bottom right), in which we did not observe any signal above the background obtained with secondary antibodies when used alone (data not shown).

After 5 minutes of incubation with BSE brain homogenates, prion-positive signals were concentrated in the subapical compartment (SAC) of Caco-2/TC7 cells, just beneath the apical brush-border domain stained with F-actin (Figure 4A), where bovine prion appeared as 400 nm to 1 μ m large vesicular structures (see arrows). Prion staining was never found above the top of the brush border, demonstrating that observed prion vesicles corresponded to endocytosed prion, and not to prion particles that would have been nonspecifically adsorbed on the apical surface of the cells. Positive vesicles present in the SAC after 5 minutes of incubation were counted on five fields, containing 400 cells each, and compared between infectious and noninfectious conditions or when using the secondary antibodies alone. F-actin staining of the apical brush-border was performed to delineate the SAC. The amount of positive vesicles in the SAC of cells incubated with BSE brain homogenates was 8- to 10-fold greater than the background detected in cells incubated with NIHs (Figure 4A, NIH conditions) or when fluorescence was analyzed using secondary antibodies alone (data not shown).

The SAC being enriched in endosomal compartments in epithelial cells,²⁹ we compared the subcellular distribution of

endocytosed bovine prion with that of early endosome antigen-1 (EEA1), a well known marker of early endosomes structures.³⁰ We observed co-localization between prion and EEA1 in the SAC as early as after 1 minute (not shown) and 5 minutes (Figure 4B, arrowheads) of incubation with bovine prion. No more co-localization with EEA1 was observed after 10 minutes (not shown). Surprisingly, the prion signal was only detected by immunofluorescence during the first 15 to 30 minutes of infection. It was no more visualized after 3 hours or 24 hours of incubation (Figure 4C), while Western blot analysis still detected PrP^{Sc} (Figure 4D) in increasing amounts (Figure 1B) at these incubation times. Such a discrepancy between immunofluorescence and biochemical data could be due to an impairment of the recognition of the epitope that may result from topological changes of the antigen when prion protein enters late endosomal/lysosomal compartments. We thus analyzed *in vitro* the staining by 8G8 of bovine PrP^{Sc} prepared from BSE brain homogenates in different pH buffers, using a dot-blot technique that allows the detection and quantification of the prion signal from nondenatured protein extracts, in similar conditions as it occurs by immunofluorescence. As expected, 8G8 recognized native bovine PrP^{Sc} at pH 7, but the signal was strongly diminished at pH 5.5, which corresponds to the lysosomal pH (Figure 4E).³¹ Such a result was not due to the fact that the lower pH has aggregated or destroyed the prion protein because, at pH 5.5, prion was still recognized by SAF32 antibody (Figure 4E), which unfortunately cannot be used for immunofluorescence studies because it does not discriminate between the endogenous PrP^C and PrP^{Sc} (Figure 3A). Consistently, no co-localization was revealed between bovine PrP^{Sc} detected with 8G8 antibody and the lysosome-associated membrane protein 2 (LAMP2) marker³⁰ (Figure 4F) after 15 minutes of prion infection, ie, when co-localization between PrP^{Sc} and EEA1 had almost disappeared. Interestingly, a large number of PrP^{Sc}-containing vesicles (white arrowheads) were very close to LAMP2-positive vesicles (empty arrowheads) and the two sets of vesicles seemed to make close contacts.

Prion Endocytosis in Caco-2/TC7 Cells Is Mediated by the 37-kD/67-kD Laminin Receptor (LRP/LR)

The laminin receptor LRP/LR was demonstrated to be required for the endocytosis of PrP^C,^{16,17,32} for PrP^{res} propagation in neuronal cells¹⁵ and for binding of PrP²⁷⁻³⁰ to mammalian cells (S. Gauczynski et al, sub-

Figure 5. Prion endocytosis in Caco-2/TC7 cells is mediated by the 37 kDa/67 kDa laminin receptor. **A:** Western blot analysis of LRP/LR expression in Caco-2/TC7 cells using anti-LRP/LR antibody W3. The band at 37 kDa corresponds to the laminin receptor precursor protein (37 kDa LRP), and the faint band at 67 kDa corresponds to the mature high-affinity laminin receptor protein (67 kDa LR). **B:** Confocal immunofluorescence analysis of LRP/LR subcellular distribution in Caco-2/TC7 cells (red channel, LRP/LR; green channel, F-actin) at apical, subapical, and perinuclear levels and XZ projection of confocal acquisitions. **Dashed gray lines** represent the lateral cell-cell junctions and the basal pole of the cells. **C:** Immunofluorescence analysis of prion-containing vesicles (red channel, 8G8) and LRP/LR (green channel, anti-LRP/LR W3) in the subapical compartment of Caco-2/TC7 cells after 5 minutes of incubation with noninfectious (NIH) or BSE brain homogenates in the apical medium. **Arrowheads** and selected frames (**insets**) show some of the numerous bovine prion and LRP/LR co-localizations in cells incubated with BSE brain homogenates. **D:** Quantification of 8G8-associated immunofluorescence in the subapical compartment of Caco-2/TC7 cells after 5 minutes of incubation with infectious (BSE, **black bars**) or noninfectious (NIH, **white bars**) brain homogenates in the apical medium and with (**dotted bars**) or without preincubation for 2 hours with 20 μ g/ml of blocking anti-LRP/LR antibody W3 or after 5 minutes of incubation with infectious brain homogenates and preincubation with 20 μ g/ml nonblocking anti-integrin-type laminin receptor (VLA-6) antibody (**hatched bar**) used as a control. In each condition, prion-positive vesicles were counted on five fields containing 400 cells each, in three independent experiments. Results are expressed as percentage of 8G8-stained vesicles found in each incubation condition, the value obtained for cells incubated with BSE brain homogenates for 5 minutes without preincubation with antibodies being set at 100%. Scale bars: 20 μ m (**B**); 10 μ m (**C**).

mitted). We investigated whether bovine PrP^{Sc} endocytosis in Caco-2/TC7 cells was mediated by LRP/LR, which was reported to be apically expressed in human enterocytes *in vivo*.^{6,14} Western blot analysis demonstrated that the two characteristic 37 kDa and 67 kDa isoforms of LRP/LR are expressed in Caco-2/TC7 cells (Figure 5A). Confocal microscopy analysis revealed that LRP/LR was mainly expressed at the base of the brush-border microvilli, but also in the SAC and at the perinuclear level (Figure 5B). After 5 minutes of incubation with BSE brain homogenates, we observed in the SAC numerous positive vesicles in which endocytosed bovine PrP^{Sc} co-localized with LRP/LR (Figure 5C, BSE conditions, see insets and arrowheads). In contrast, very few fluorescent vesicles were observed after incubation with noninfectious brain homogenates (Figure 5C, NIH conditions), which in fact corresponded to the background signal obtained when using the secondary antibody alone (not shown). To demonstrate the involvement of LRP/LR in the internalization of bovine prion, we studied in our model the impact of the W3 antibody, which was reported to block LRP/LR activity in neurons.¹⁵ We show that preincubation of Caco-2/TC7 cells with W3 anti-LRP/LR blocking antibody had no effect when cells were incubated with NIH but resulted in a significant decrease of PrP^{Sc} endocytosis when cells were incubated with BSE-infected brain homogenates (Figure 5D). By contrast, preincubation of the cells with an anti-integrin-type laminin receptor (VLA-6) antibody did not induce any change in the uptake of prion particles in cells incubated with BSE-infected brain homogenates (Figure 5D).

Discussion

We report here for the first time that bovine prion is internalized in human Caco-2/TC7 enterocytes, via an LRP/LR-mediated endocytosis. This supports the hypothesis that enterocytes, the major cell population of the intestinal epithelium, play a role for the uptake of prion infectious particles during oral infection. It has been proposed⁸ that *trans*-epithelial prion transport in intestine could occur through M cells, a rare type of epithelial cells located in the covering epithelium of Peyer's patches. These M cells are specialized in phagocytosis of infectious particles from intestinal lumen, and their subsequent delivery to immune cells. Prion infectivity was detected in the basal compartment of model M-like cells,⁸ obtained by co-culturing Caco-2/TC7 cells and lymphocytes,³³ after an apical supply with Rocky Mountain Laboratory mouse scrapie strain prions.³⁴ It is conceivable that such a process might be initiated through a nonspecific phagocytosis, as demonstrated for other pathogens or microorganisms in M cells.^{35,36}

Our results showed that bovine PrP^{Sc} from brain extracts of BSE-contaminated cows can be internalized and accumulated into Caco-2/TC7 cells that had maintained characteristics of enterocytes and were not converted into M-like cells, as demonstrated by the maintenance of the expression of the specific enterocyte brush border-associated marker sucrase-isomaltase. Interestingly, pri-

ons from brain extracts of mouse-adapted scrapie strain C506M3 that is taken up by murine intestinal cells⁵ were not internalized in Caco-2/TC7 cells, suggesting a specific uptake of bovine BSE prion by human enterocytes, in accordance with the well-known capacity of the BSE strain to cross interspecies barriers. Although BSE brain homogenates prepared as scrapie-associated fibrils (SAFs) are known to be highly infectious in nerve cells,²⁵ we did not detect any internalization of bovine prion in Caco-2/TC7 cells apically supplied with BSE-SAFs. This result might be explained by topological constraints, macromolecular fibrils being too large to be processed by specific endocytosis, whereas they could be easily processed by nonspecific phagocytosis in M cells.⁸ Furthermore, it must be emphasized that the tissue homogenates that we used in our experiments are closer to the form of bovine prion supplied by contaminated meat during oral infection than to purified SAFs.

Interestingly, though prions exhibit serious deleterious effects on nerve cells,¹ bovine PrP^{Sc} did not induce any deleterious effect on Caco-2/TC7 cell shape. Accordingly, dendritic cells or macrophages display no cell damage although they contribute to PrP^{Sc} spreading and are considered as prion reservoirs, due to their high content in PrP^C available for transconformation.³⁷ One could wonder whether in enterocytes, as in immune cells, bovine prion could initiate endogenous PrP^C transconformation into PrP^{Sc}. Because cell polarity and cell-cell junctions were not perturbed by the internalization of bovine prion in Caco-2/TC7 cells, endogenous PrP^C remained targeted to lateral cell-cell junctions of enterocytes (Figure 2C)²² and its absence in the apical brush-border membrane precluded any direct interaction between apically supplied bovine PrP^{Sc} and endogenous PrP^C. Furthermore, no transconformation of PrP^C had occurred during the early steps of prion infection in Caco-2/TC7 cells because we have not detected any human PrP^{Sc}, although, once endocytosed, bovine PrP^{Sc} might have interacted with the endogenous trafficking/recycling PrP^C in the subapical compartment (SAC).

Endocytosis and intracellular trafficking of pathological prion and/or PrP^{Sc} isoform are markedly less understood and documented than that of endogenous PrP^C. The latter, as other GPI-anchored proteins, is rapidly recycled from the plasma membrane, probably via clathrin-mediated³⁸ or caveolae-mediated³⁹ endocytosis. These two major pathways to (re)enter cells are directly connected to intracellular organelles^{30,40} and can be used for pathogen entry, notably viruses.⁴⁰ PrP^C has been observed along the endocytic pathway in nerve cells.¹⁹ Some studies put forward the concept that PrP^{Sc} probably follows the same endocytosis process as its cellular counterpart, and the presence of PrP^{Sc} had been reported in late endosomes in scrapie-infected mouse brains.^{19,41} Using 8G8 antibody that discriminates the bovine PrP^{Sc} from the human endogenous PrP^C, we demonstrated that bovine prion is rapidly endocytosed by Caco-2/TC7 cells, and accumulates as 8G8-positive vesicular structures in the subapical compartment (SAC) of cells. Moreover, the comparison of the subcellular distribution of endocytosed prion with that of the early endosome marker (EEA1)

revealed that bovine prion is trafficked during the first 5 minutes of incubation to early endosomes, a major sorting structure in the endocytic pathway.³⁰ Prion vesicles co-localized with endogenous LRP/LR in Caco-2/TC7 cells, indicating that both proteins are trafficked in the same vesicular compartment at this early step of infection. It has been proposed that LRP/LR acts as the PrP^C receptor at the cell surface^{15–18,20} (and as a receptor for PrP27-30; S. Gauczynski et al, submitted). As already reported for enterocytes *in vivo*,^{6,14} we showed the presence of LRP/LR in the apical brush-border of Caco-2/TC7 cells and we observed a decreased uptake of bovine prion particles after preincubation of the cells with the blocking anti-LRP/LR antibody W3 but not after preincubation with the anti-integrin-type laminin receptor (VLA-6) antibody (Figure 5). Altogether, these results allow us to propose that bovine prion was taken up from the apical compartment through LRP/LR-dependent endocytosis and trafficked to early endosomal structures in Caco-2/TC7 enterocytes.

The 8G8 immunofluorescence signal disappeared in contaminated cells after 15 to 30 minutes of prion incubation (Figure 4C), whereas bovine prion accumulation was still observed in cell lysates (Figure 4D), questioning the epitope recognition of bovine PrP^{Sc} by 8G8 antibody along the endocytic pathway. Indeed, 8G8 monoclonal antibody recognizes the 95 to 110 peptide,²⁸ contiguous to the octapeptide repeats (OPR) region (amino acids 51 to 91), and it has been demonstrated that PrP conformation and folding are pH-dependent,^{42,43} notably the N-terminal region of PrP that contains this OPR domain.^{44,45} We showed that prion detection by 8G8 antibody was strongly diminished in lysosomal-like pH buffers. This could explain the lack of co-staining of 8G8 prion vesicles with LAMP2 lysosomal marker. However, we also clearly observed close vicinity between prion-vesicles and late-endosome/lysosome structures, which suggests that PrP^{Sc} was targeted to lysosomes after LRP/LR-mediated endocytosis.

In conclusion, our results sustain the hypothesis of a specific route for internalization of prion particles in human intestinal cells after oral ingestion of bovine prion-infected tissues, a hypothesis first proposed by Shmakov and colleagues.⁶ In addition to nonspecific phagocytosis by M cells,⁸ our results underline a potential role of enterocytes, the major cell population of the intestinal epithelium,¹¹ even at the level of Peyer's patches,¹² in the absorption of prions during oral infection through a specific LRP/LR receptor-mediated process.

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