The Cellular Prion Protein PrP\textsuperscript{c} Is Expressed in Human Enterocytes in Cell-Cell Junctional Domains*

Received for publication, August 5, 2003, and in revised form, October 1, 2003
Published, JBC Papers in Press, October 23, 2003, DOI 10.1074/jbc.M308578200

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The physiological function of PrP\textsuperscript{c}, the cellular isoform of prion protein, still remains unclear, although it has been established, \textit{in vitro} or by using nerve cells, that it can homodimerize, bind copper, or interact with other proteins. Expression of PrP\textsuperscript{c} was demonstrated as necessary for prion infection propagation. Considering the importance of the intestinal barrier in the process of oral prion infectivity, we have analyzed the expression of PrP\textsuperscript{c} in enterocytes, which represent the major cell population of the intestinal epithelium. Our study, conducted both on normal human intestinal tissues and on the enterocytic cell line Caco-2/TC7, shows for the first time that PrP\textsuperscript{c} is present in enterocytes. Interestingly, we found that this glycosylphosphatidylinositol-anchored glycoprotein was localized in cholesterol-dependent raft domains of the upper lateral membranes of enterocytes, beneath tight junctions, in cell-cell junctional domains. We observed that PrP\textsuperscript{c}, E-cadherin, and Src co-localized in adherens junctions and that PrP\textsuperscript{c} was co-immunoprecipitated with Src kinase but not with E-cadherin. Alteration of cell polarity after cholesterol depletion or loosening of the cell-cell junctions after EGTA treatment rapidly impaired membrane targeting of PrP\textsuperscript{c}. Overall, our results point out the signaling of cell-cell contacts as a putative role for PrP\textsuperscript{c} in epithelial cells.

The cellular prion protein (PrP\textsuperscript{c}) was identified essentially because of its involvement in infectious degenerative encephalopathies (1). Indeed, the expression of PrP\textsuperscript{c} has been demonstrated as required for prion infection propagation (2, 3), and infectivity has been suggested to be the consequence of conformational modification of PrP\textsuperscript{c} by the infectious prion protein scrapie (PrP\textsuperscript{Sc}) (1). Efforts were mainly directed at understanding infection and have essentially focused on nerve cells. The biological roles of PrP\textsuperscript{c} still remain unclear. It has been established, however, that PrP\textsuperscript{c} can homodimerize (4), interacts with other proteins, such as synapsin Ib, Grb2, Pint1, LRP/LR, and N-CAMs (5–12), and binds copper (13). It has also been suggested (14) that PrP\textsuperscript{c} triggers cell signaling through interaction with phosphorylated Fyn. In addition, it has been shown that multiple biochemical changes occurred in prion protein knockout mice. They included increased levels of NF-\textit{B} and COX-IV and decreased levels of p53 and Cu,Zn superoxide dismutase activity, along with an increased neuronal sensitivity to oxidative stress in cultured cells isolated from these knockout mice (15).

Although oral transmission for prions diseases has been demonstrated, the site at which the infectious agent crosses the intestinal epithelium is still debated. After oral infection, the rapid accumulation of PrP\textsuperscript{Sc} in Peyer’s patches (16, 17) led to the concept that M cells, which are present in the covering epithelium of lymphoid follicles, were responsible for the intestinal transfer of prion infectious particles toward the immune system (18, 19). Indeed, M cells have a high activity of endocytosis and transport a wide variety of macromolecules and microorganisms to the mucosa-associated lymphoid tissue (20). However, enterocytes represent the major cell population of the intestinal epithelium (21), even over Peyer’s patches (22), and PrP\textsuperscript{Sc} has also been detected in the enterocytes of the villous epithelium of the small intestine of primates after oral infection (23).

While being a prerequisite for prion replication in nerve cells, rare studies have dealt with the expression of the normal cellular counterpart PrP\textsuperscript{c} in the gastrointestinal tract, where replication might also occur. In these reports, the presence of high levels of PrP\textsuperscript{c} was observed in crypts (24), in the mucus of some rare goblet cells (25), in the intestinal vascular endothelium (26), in afferent nerves of the lamina propria, and in the dispersed neuroendocrine system (27). However, the presence of PrP\textsuperscript{c} in enterocytes has not been specifically investigated.

To determine whether enterocytes express PrP\textsuperscript{c}, we used normal human intestinal tissues and the Caco-2/TC7 cell model that we have developed and that is morphologically and functionally similar to normal human enterocytes (28). Our results showed that PrP\textsuperscript{c} is present in enterocytes and, interestingly, that this glycosylphosphatidylinositol (GPI)-anchored protein is targeted to junctional complexes, in the lateral membranes of adjacent cells, where it interacts with Src kinase. These results open new directions for evaluating the biological function of PrP\textsuperscript{c} in epithelial cells.

**EXPERIMENTAL PROCEDURES**

Reagents—All chemicals were purchased from Sigma except where indicated. Mouse 12F10 (against peptide 142–160) and SAF32 (against...
peptide 79–92) (29, 30) anti-human PrPc monoclonal antibodies were obtained from the laboratory of J. Grassi (Service de Pharmacologie et Immunologie, CEA, Saclay, France). Rabbit anti-human ZO1 polyclonal antibodies, mouse HEC1D anti-human E-cadherin monoclonal antibody, and rat anti-mouse E-cadherin monoclonal antibody (ECCD-2) were purchased from Zymed Laboratories Inc. Rabbit anti-human E-cadherin (HB-108), anti-human Src (sc-18), and anti-human PrPc (FL-253) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Src monoclonal antibody (clone GD11) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit L459 anti-human sucrase isomaltase polyclonal antibodies were used as described previously (28). Secondary CY2-, CY3-, and CY5-labeled antibodies were purchased from Jackson Immunoresearch (West Grove, PA). F-actin was labeled with phalloidin-fluorescein isothiocyanate.

Cell Culture and Human Tissues—Caco-2/TC7 cells (28) were cultured on 1-μm pore-size microporous PET filters (Falcon, BD Biosciences) in a 10% CO₂ atmosphere in high glucose Dulbecco’s modified Eagle’s medium Glutamax 1 (Invitrogen) supplemented with 20% heat-inactivated fetal calf serum (AbCys, Paris, France), 1% non-essential amino acids (Invitrogen), penicillin (100 IU/ml), and streptomycin (10 μg/ml) (Invitrogen). In some experiments cells were cultured in plastic flasks (Falcon) or on glass lamellae (Polylabo, Strasbourg, France). SH-SY5Y human neuroblastoma cells were grown on 10-cm diameter plastic dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM nonessential amino acids, and 50 units/ml penicillin/streptomycin (Invitrogen) in a 5% CO₂ humidified incubator at 37°C. Normal ileum and jejunum mucosa samples were collected from irreversibly brain-damaged kidney donors (obtained from France Transplant).

Cell Treatments—For cholesterol depletion, cells were cultured for 48 h in Dulbecco’s modified Eagle’s medium Glutamax 1 supplemented with a lipoprotein-deficient serum and mevastatin (10 μM). β-Cyclodextrin (2.5 mM) was added for the last 18 h of culture (31, 32). The cellular junctional tightness was perturbed by treatment with EOTA (2 mM) for 6, 10, 15, and 30 min and monitored by apical biotin labeling. At each indicated time, cells were washed with cold PBS (Invitrogen) and immediately fixed for 30 min, at 4°C, with 4% paraformaldehyde (PFA) in PBS.

Confocal Fluorescence Microscopy—Cells cultured on filters or lamellae were fixed for 30 min with 4% PFA in PBS and permeabilized for 20 min with 0.1% Triton X-100 in PBS. Frozen human tissue samples were embedded in tissue-Tek (Dako) and cut with a cryo-microtome (Leica, Rueil Malmaison, France). Unlike cultured cells, human tissue sections were not permeabilized after treatment with 4% PFA. Cultured cells and human tissue sections were examined by confocal fluorescence microscopy (Zeiss LSM 510).

RESULTS

PrPc Is Expressed in Human Enterocytes and Concentrated in Cell-Cell Junctional Complexes—We analyzed the presence...
Fig. 2. Expression and localization of PrPc in Caco-2/TC7 cells. A and B, confocal microscopy images of PrPc localization in polarized/differentiated (A) and exponentially growing (B) Caco-2/TC7 cells (12F10 antibody (Ab)). White arrows indicate PrPc localized at cell-cell contact domains, and arrowheads indicate lateral membranes devoid of PrPc in the absence of cell-cell contacts. Scale bars, 20 μm. C, confocal microscopy image of PrPc in Caco2/TC7 cells after staining with 12F10 antibody saturated with the antigenic peptide. Scale bar, 20 μm. D, confocal microscopy image of PrPc localization in Caco2/TC7 cells after staining with SAF32 antibody. Scale bar, 20 μm. E, Western blot analysis of PrPc expression pattern in exponentially growing (day 3 lanes, 50 μg of protein) and in polarized/differentiated (day 11 lanes, 50 μg of protein) Caco-2/TC7 cells. Membranes were revealed with 12F10 antibody (left panel), 12F10 antibody saturated with the antigenic peptide 142-260 (middle panel), or SAF32 antibody (right panel). F, Western blot analysis of PrPc amounts in polarized/differentiated Caco2/TC7 cells and in SH-SY5Y nerve cells (40 μg were loaded in each lane). After transfer, proteins were stained with Ponceau Red, and PrPc was revealed with SAF32 antibody. Scanning was performed using the ImageQuant 5.1 software (Amersham Biosciences). PrPc level in each cell type (arbitrary units) corresponds to the ratio between the PrPc-specific and the total staining signals. Results shown are representative of two independent experiments.

and the distribution of PrPc in normal human intestinal tissues. Our results revealed that PrPc is present throughout the epithelium, in villi, where it is concentrated below the apical brush-border (Fig. 1A and B), and in the crypt-villus junctions (Fig. 1C), in cells that express sucrase-isomaltase, a specific marker for the brush-border of enterocytes. PrPc was never observed in the apical membrane of the brush-border microvilli and, accordingly, did not co-localize with sucrase-isomaltase (Fig. 1C). In fact, PrPc was found mostly concentrated beneath the tight junction-associated protein zonula occludens 1 (ZO1) (Fig. 1B). Moreover, the optimal cut incidence regularly obtained in crypt sections revealed that PrPc was concentrated in the upper part of the lateral membrane at the site of contact between two adjacent cells (Fig. 1C). We also found high levels of PrPc in the mucus of a few goblet cells (not shown) and in the vascular endothelium in the lamina propria (Fig. 1A), as reported in previous studies (24–27) that did not detect PrPc in enterocytes.

We then analyzed the distribution of PrPc in enterocytic Caco-2/TC7 cells cultured on microporous filters. In confluent and polarized Caco-2/TC7 cells, PrPc was concentrated in the lateral membrane (Fig. 2A and inset). Conversely, a high proportion of the protein remained intracellularly distributed in exponentially growing cells (Fig. 2B). However, in this last condition, a small proportion of PrPc was already located at the plasma membrane within the expanding cell clusters. Interestingly, the localization of PrPc at the lateral membrane appeared to be dependent on cell-cell contacts (Fig. 2B, arrows) as no PrPc labeling was observed, before confluence, on membranes not committed to cell-cell adhesion, i.e. around cell clusters (Fig. 2B, arrowheads). The specificity of the PrPc signals was assessed by using 12F10 antibody previously saturated with the 142–160 antigenic peptide (Fig. 2C). Moreover, SAF32 antibody gave similar PrPc signals at the lateral membrane in confluent Caco-2/TC7 cells (Fig. 2D). Western blot analysis, using 12F10 or SAF32 antibody, showed that a similar amount of PrPc was present in exponentially growing and confluent polarized cells (Fig. 2E). Several bands were obtained around 35 kDa, the specificity of which was controlled after blotting with 12F10 antibody previously saturated with the 142–160 antigenic peptide (Fig. 2E, middle panel). The amount of PrPc was compared by Western blot in confluent polarized Caco-2/TC7 cells and in SH-SY5Y nerve cells. Results reported in Fig. 2F showed that equal amounts of PrPc were present in both cell types. Moreover, the pattern of the isoforms detected with SAF32 antibody differed between Caco-2/TC7 and SH-SY5Y cells, most probably due to changes in the respective proportions of the glycosylated isoforms.

As observed in the normal human intestinal epithelium, PrPc was localized below ZO1 in the lateral membrane of Caco-2/TC7 cells (white arrows, Fig. 3A), in cell-cell contacts domains. PrPc and ZO1 never co-localized. By using immunoelectron microscopy (Fig. 3B), we showed that PrPc was associated with specialized domains of the lateral membrane, identified as junctional complexes with a thickening of the adjacent plasma membranes and their association with cytoskeleton. These junctional complexes were further shown to be adherens junctions as PrPc, E-cadherin, the main component of these junctions, and F-actin gave a merged signal in confocal microscopy at the major expression plane of PrPc (Fig. 3C).

Cholesterol-dependent Membrane Targeting of Mature PrPc in Caco-2/TC7 Cells—By cholesterol depletion (Fig. 4), using mevastatin and β-cyclodextrin (32), we further confirmed that PrPc is localized in rafts microdomains of enterocytes, as in other cell types (33). Although cholesterol depletion did not affect cell viability, it did induce a loss of the polarization of
Caco-2/TC7 cells as shown by cell rounding and the delocalization of sucrase isomaltase on the whole cell membrane (not shown). As expected, ZO1, which is associated with the tight junctions known to be raft domains (34), was redistributed in the cytoplasm. In these conditions, PrPc was detected intracellularly rather than at the lateral membrane, demonstrating that the membrane targeting of PrPc requires the integrity of cholesterol-dependent raft microdomains.

**Perturbation of Junctional Complexes Interferes with the Membrane Targeting of PrPc**—We then analyzed whether perturbation of junctional complexes affects the membrane localization of PrPc. Loosening of cell-cell adhesion in confluent and polarized Caco-2/TC7 cells, following 2 mM EGTA-mediated Ca\(^{2+}\) depletion (35), resulted in a progressive permeability of the monolayer to apically delivered biotin (data not shown) and, as expected (35), in the internalization of E-cadherin (Fig. 5A) without affecting the cell shape and the association of ZO1 to the tight junctions (Fig. 5B). In these conditions, PrPc was no longer detected at the lateral membrane (Fig. 5, A and B), and it was found exclusively intracellularly.

**Membrane PrPc Interacts with Src Kinase**—Src kinase is known to be targeted to cadherin-dependent junctions (36), and PrPc has been reported to interact with Fyn (14), a member of the Src family kinases, in neuronal cells. Therefore, we con-
pared the distribution of PrPc and Src kinase in confluent and polarized Caco-2/TC7 cells. Confocal microscopy showed a co-localization of both proteins at the cell-cell contacts (Fig. 6A). Immunoelectron microscopy analysis of the co-localization zones revealed that PrPc and Src were regularly in the immediate vicinity one of the other in these zones (Fig. 6B). The measured distance between PrPc and Src, 12.5 nm in majority, was compatible with the 10-nm thickness of the membrane, PrPc being anchored in the external leaflet while Src is anchored in the internal one. The observation of a potential interaction between PrPc and Src was further supported by biochemical data. Detergent-treated cell lysates were subjected to sucrose density gradient experiments, and the resulting fractions were analyzed for the presence of PrPc, Src, and E-cadherin. PrPc and E-cadherin were recovered both in the detergent-insoluble and detergent-soluble fractions, whereas Src was mainly found in the detergent-insoluble fraction (Fig. 6C). The detergent-insoluble fraction was then subjected to immunoprecipitation experiments with antibodies directed against PrPc or against Src kinase. We found that PrPc and Src interacted, whereas PrPc and E-cadherin did not. Indeed, a pool of Src was co-immunoprecipitated with anti-PrPc antibodies and vice versa (Fig. 6D). In contrast, E-cadherin, which is known to interact with Src (36), was co-immunoprecipitated with anti-Src antibodies but not with anti-PrPc antibodies.

**DISCUSSION**

This report is the first demonstration that PrPc is expressed in enterocytes (Fig. 1), which account for 80% of the intestinal epithelial cells (21). The amount of PrPc expressed in human Caco-2/TC7 enterocytes was similar to that of human SH-SY5Y nerve cells (Fig. 2). Interestingly, in the human intestine and in differentiated enterocytic Caco-2/TC7 cells, this GPI-anchored protein is targeted to junctional complexes of the lateral membranes of adjacent polarized cells. PrPc was found in cholesterol-dependent rafts in adherens junctions where it co-localized with...
E-cadherin, the major component of these junctional complexes (Fig. 3). However, the observation that PrPc and E-cadherin were not co-immunoprecipitated excludes the possibility that they interact or participate in the same protein complex.

GPI-anchored proteins are considered targeted to the apical membrane domains of polarized cells. However, the localization of a GPI-anchored protein in junctional domains has already been observed for T-cadherin, a cell-cell binding signaling protein (37). Our finding in cells that endogenously express PrPc was further supported by Sarntatro et al. (38) who reported that a transfected PrPc was targeted to the basolateral membrane of Madin-Darby canine kidney and PRT epithelial cells. Moreover, we also observed that PrPc exhibited the same localization in normal human keratinocytes as in enterocytes, i.e. at the junctions between adjacent cells (data not shown). Our results open questions about the role that PrPc can play such as the Gα subunit and Src family kinases (37). Our results demonstrate that PrPc co-immunoprecipitates with a pool of Src kinase (Fig. 6) while E-cadherin co-immunoprecipitates with another one. Therefore, it may be hypothesized that PrPc plays a role in intercellular signaling and/or sensing of neighboring cells, through an interaction with Src kinases, rather than in adhesion per se. Because PrPc is anchored in the outer membrane and Src in the inner one, such a PrPc-dependent signaling does involve an intermediate factor(s) that has to be characterized. This intermediate partner of the PrPc-Src complex cannot be the transmembrane adhesion molecule E-cadherin since PrPc and E-cadherin were independently co-immunoprecipitated with two different pools of Src kinases (Fig. 6). The precise localization of PrPc in junctional complexes suggests that it could play a distinct role in epithelial cells and in nerve cells where this protein is present on the whole cell surface, binds to N-CAM (10), and was suggested to trigger signaling through the Src kinase Fyn activation (14). Combined with our results, this could suggest that PrPc plays a role in cell-cell adhesion signaling in epithelial cells and in cell-matrix adhesion signaling in nerve cells. Src kinases are known to be involved in both signaling pathways, being targeted to integrin-dependent cell-matrix and to cadherin-dependent cell-cell junctions where they phosphorylate substrates that induce adhesion turnover and actin remodeling (36).

Another important result is that alteration of the cell monolayer integrity (i.e. cholesterol depletion or loosening of cell junctions) leads to the intracellular sequestration of PrPc (Figs. 4 and 5), suggesting that the targeting of PrPc to junctional complexes can be modulated according to the physiological state of the epithelium. Whether similar modulation of the membrane targeting of PrPc also occurs in pathological conditions remains to be determined. Interestingly, Hepner et al. (18), using Caco-2/TC7 cells, reported that infectious scrapie prion units are more easily transferred through the cell monolayer when cells have acquired an M cell phenotype resulting from a co-culture with B lymphocytes. In view of our results, it may be hypothesized that the drastic cell remodeling that accompanies the enterocyte to M cell transition in this model, i.e. a disassembly of the brush border and a redistribution of villin to the cytoplasm (41), may have induced the intracellular sequestration of PrPc and favored intracellular interactions between PrPc and the endogenous PrPc. However, the presence and/or distribution of endogenous PrPc in untreated Caco-2/TC7 cells or in the “M” cells resulting from co-culture were not analyzed. These results do not preclude that a transfer of infectious particles, although less abundant than in M cells since it must involve an active transcellular transport, could also occur in the initial Caco-2/TC7 enterocytes.

In conclusion, the results reported here point out the signaling of cell-cell contacts as a putative role for the endogenous cellular prion protein PrPc in epithelial cells, through interaction with Src kinase. In addition, the human enterocty Caco-2/TC7 cell line appears as an accurate model to investigate whether and how enterocytes participate in the passage of infectious particles through the intestinal epithelium, and whether these cells could represent a site of PrPc transconformation by the pathogenic prion agents.

Acknowledgments—We thank Jean-Luc Olivier and our colleagues from U505 for helpful discussions. The confocal and electron microscopy analyses were performed using the facilities of IFR 95.

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