Cell. Mol. Life Sci. (2015) 72:1185–1196
DOI 10.1007/s00018-014-1735-8

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Prion strains are differentially released through the exosomal pathway

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Received: 7 May 2014 / Revised: 20 August 2014 / Accepted: 12 September 2014 / Published online: 18 September 2014

Abstract Cell-to-cell transfer of prions is a crucial step in the spreading of prion infection through infected tissue. At the cellular level, several distinct pathways including direct cell–cell contacts and release of various types of infectious extracellular vesicles have been described that may potentially lead to infection of naïve cells. The relative contribution of these pathways and whether they may vary depending on the prion strain and/or on the infected cell type are not yet known. In this study we used a single cell type (RK13) infected with three different prion strains. We showed that in each case, most of the extracellular prions resulted from active cell secretion through the exosomal pathway. Further, quantitative analysis of secreted infectivity indicated that the proportion of prions eventually secreted was dramatically dependent on the prion strain. Our data also highlight that infectious exosomes secreted from cultured cells might represent a biologically pertinent material for spiking experiments. Also discussed is the appealing possibility that abnormal PrP from different prion strains may differentially interact with the cellular machinery to promote secretion.

Keywords Prion release · Cell models · Exosome · Extracellular vesicle · Prion spiking · TSE agent

Introduction

Prions are infectious agents responsible for fatal neurodegenerative diseases named transmissible spongiform encephalopathies (TSE) that affect various animal species, including humans [1]. Prions are thought to be at least

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partly composed of abnormally folded conformers (designated PrP^{sc}) derived from the normal cellular PrP protein (PrP^{c}). The aggregated, abnormally folded PrP^{sc} species multiply by recruiting and converting host PrP^{c} into new PrP^{sc} [2], a seeding process that can be reconstituted in test tubes by a process known as protein misfolding cyclic amplification [3].

Prions transmit among individuals of the same or different species [4] and spread within the organism from peripheral tissues to the brain [5], where damage is observed. As a result, cell-to-cell spreading (the ability of prions from one cell to infect another) is a critical feature of the prion life cycle. As a first step towards identifying the mechanisms by which cell-to-cell spreading occurs in vivo, cell culture experiments indicate that prions may use strikingly different pathways to infect new cells [6]. Direct cell contacts may trigger the infection of naïve cells [7–9]. The underlying mechanism may involve trans interactions of PrP^{sc} and PrP^{c} in the closely apposed plasma membranes of infected and naïve cells, respectively. Alternatively, intracellular PrP^{sc} could be transferred directly to recipient cells though connecting tunnelling nanotubes [10]. On the other hand, the long-lasting observation that culture medium from infected cells is infectious led to the demonstration that infected cells do release vesicle-associated prions into the extracellular space. Extracellular vesicles (EV) harbouring prions include shedding vesicles (large vesicles up to 1 µm, budding from the plasma membrane) [11] and exosomes (50- to 100-nm vesicles formed in the endosomal pathway and released upon fusion of multivesicular endosomes with the plasma membrane) [12–14]. Interestingly, recent studies have pointed to exosomes as being vehicles for the cell-to-cell transfer of several other misfolded proteins associated with neurodegenerative diseases [15–17].

The efficiency of prion secretion and the relative contribution of the different EV types (shedding vesicles, exosomes) to extracellular prions have not been precisely quantified due to the large number of animals required for bioassays of prions. In this study, we used a recently developed cell-based assay [18] to quantitatively investigate prion release from RK13 cells infected with prion strains from three different species. In this cell model, our quantitative analysis showed that exosomal release was the main pathway for prion secretion. Strikingly, we found that depending on the prion strain the proportion of intracellular prions eventually secreted varied from ~0.1 to ~3 %. This finding raises the intriguing possibility that different abnormal PrPs differentially interact with the cellular machinery responsible for exosome secretion.

Materials and methods

Cells and prion agents

The generation of RK13 cell clones expressing the ovine PrP^{c} (ovRK13, clone Rov9) or the mouse PrP (moRK13, clone mo1) has been described previously [18, 19]. The cell clone expressing the bank vole PrP^{c} (voRK13, clone Cg6) used in this work was selected after transfection of RK13 cells with a pTRE-based expression vector encoding the bank vole PrP^{c} (methionine at codon 109), as described [20].

Ovine 127S prion is the sheep isolate (PG127, [21]), propagated once in sheep and amplified in *tg338* mice [22]. The mouse strain 22L was propagated in C57BL/6 mice. The bank vole strain was cattle BSE adapted (3 passages) to methionine 109 bank voles [23, 24]. At the terminal stage of the clinical disease, all brains were homogenized at 10 % (wt/vol) in 5 % sterile glucose using a high-speed homogenizer (TeSeE Precess 48 system).

Establishment of persistently infected cultures

The cells were maintained at 37 °C in 5 % CO_2 in Opti-MEM medium (Invitrogen) supplemented with 10 % foetal bovine serum, 100 U/mL penicillin and 10 mg/mL streptomycin. To induce the expression of ovine, mouse or vole PrP^{c}, 1 µg/mL doxycycline (dox, Sigma-Aldrich) was added to the culture medium. Dox-treated cells, grown in 12-well microtiter plates, were incubated for 3–5 days in the presence of infectious brain homogenates (0.5 % vol/vol final). Each inoculated culture was then trypsinized and grown for 1 week in a T-25 cm^2 flask. The cultures were then passaged every week at a ¼ dilution in the presence of dox until conditioned medium was prepared.

Conditioned media

The culture medium was centrifuged overnight at 100,000×*g* (g_{av}) at 4 °C in polyallomer tubes in an SW32 Ti rotor (Beckman, k factor: 204) to remove foetal bovine serum exosomes. Conditioned media and the different fractions were typically prepared from eight T-150 cm^2 flasks. Each culture was seeded in eight T-150 cm^2 flasks (~10^7 cells per flask in 25 mL of exosome-depleted cell culture medium). One week later, the cell monolayers of some flasks were trypsinized. The cells were collected by centrifugation, resuspended in a glucose solution and homogenized with a high-speed homogenizer (TeSeE Precess 48 system) for assessment of infectivity using a cell-based assay. The corresponding cell culture medium (s0K) was centrifuged at 2,000×*g* for 20 min at 4 °C. The p2K pellet was recovered and the supernatant (s2K)

centrifuged at 10,000×*g* for 30 min at 4 °C. The p10K pellet was recovered and approximately 180 mL of the supernatant (s10K) was ultracentrifuged at 100,000×*g* for 75 min at 4 °C in an SW32 Ti rotor. The supernatant (s100K) was recovered and the corresponding pellets were resuspended in a small volume of PBS, pooled in a single tube, diluted to 30–35 mL with PBS and ultracentrifuged again at 100,000×*g* for 1 h 15 min at 4 °C. The final pellet (p100K) was resuspended in a small volume of PBS and the protein content was quantified using bicinchoninic acid (BCA, Pierce). All fractions were stored at −20 °C until analysis.

Discontinuous sucrose gradients

Resuspended p100K was diluted to 2.6 mL with 2.5 M sucrose in 20 mM HEPES pH 7.4 and poured into a polyallomer SW32 Ti tube. Eight solutions of decreasing sucrose concentrations (from 2 to 0.25 M in 20 mM HEPES pH 7.4) were then successively layered (4 mL per layer). After overnight centrifugation at 100,000×*g* at 4 °C, nine fractions (4 mL each) were collected from the top. The gradient pellet was resuspended in 4 mL of PBS. The density of each fraction was measured with a refractometer. Each fraction was diluted with 30 mL of PBS and centrifuged again at 100,000×*g* for 75 min at 4 °C, and the resulting pellet was resuspended in a small volume of PBS. Alternatively, the proteins within each fraction were concentrated by methanol precipitation.

Cell-based assay of infectivity

The cell-based assay procedure has been described previously [18]. In this study, the assayed samples were conditioned media (s0K, s2K, s10K, s100K), p2K, p10K, p100K, cell homogenates and samples harvested from gradient fractions. The indicated volumes of conditioned media to be tested were diluted to 3 mL with cell culture medium containing 1 µg/mL dox. The p2K, p10K and p100K corresponding to the indicated volume of conditioned medium or to the indicated number of secreting cells was diluted to 3 mL with cell culture medium containing 1 µg/mL dox. Homogenates from the indicated number of cells were diluted to 3 mL with cell culture medium containing 1 µg/mL dox. In some experiments, samples were diluted in cell culture medium containing no dox (−dox). Samples from gradient fractions and gradient pellets were diluted to 3 mL with cell culture medium containing 1 µg/mL dox. All samples were incubated with the recipient cells (ovRK13, moRK13 or voRK13) in individual wells of six-well plates. One week later, the media were renewed. Infection was allowed to proceed for three more weeks with one medium change per week. At the end of the infection period, the cells in each well were rinsed with cold PBS and solubilized for 10 min at 4 °C in 1 mL of Triton–DOC lysis buffer (50 mM Tris/HCl pH 7.4, 0.5 % Triton-X100, 0.5 % sodium deoxycholate). The lysates were clarified by low-speed centrifugation (425×*g*, 1 min), and cellular proteins in the post-nuclear supernatants were quantified using bicinchoninic acid. Digestion of 750–1,000 µg of proteins with PK (recombinant grade, Roche) was performed for 2 h at 37 °C with a mass ratio of 4 µg of PK per mg of cellular proteins. The digestion was stopped by addition of Pefabloc (Sigma-Aldrich) to 4 mM. PK-digested samples were centrifuged for 30 min at 20,000×*g*, and the resulting pellets were analysed for PrP^{res} by Western blot.

Immunoblotting

Samples were separated by 12 % SDS-PAGE electrophoresis and electroblotted onto PVDF membranes (Bio-Rad). The Western blots were stained for PrP with Sha31 mAb [25], for flotillin-1 with flotillin-1 mAb (BD Biosciences), for Alix with H-270 pAb (Santa Cruz Biotechnology), for EF1α with anti- EF1α mAb (clone CBP-KK1, Millipore), for GM130 with 35/GM130 mAb (BD Transduction Laboratories), for Bcl2 with 7/Bcl2 mAb (BD Transduction Laboratories) or for Tom20 with FL-145 pAb (Santa Cruz Biotechnology). Filters were developed using an ECL+ reagent kit (Amersham-GE Healthcare) and visualized with a Bio-Rad VersaDoc imaging system.

Isolation of PrP^{res} in cellular and p100K fractions

Cell cultures were rinsed with cold PBS and solubilized for 10 min at 4 °C in Triton–DOC lysis buffer. The lysates were clarified by low-speed centrifugation (425×*g*, 1 min), and cellular proteins in the post-nuclear supernatants were quantified using bicinchoninic acid. Proteins in p100K were quantified by bicinchoninic acid. Each sample of cellular proteins (9, 45 and 225 µg) and p100K proteins (9 µg) was diluted to 500 µL, and the protein concentration was adjusted to 1 mg/mL by adding Triton–DOC lysate of non-infected cells. Each sample was digested with 2 µg of PK for 2 h at 37 °C and PrP^{res} was collected and analysed as indicated above.

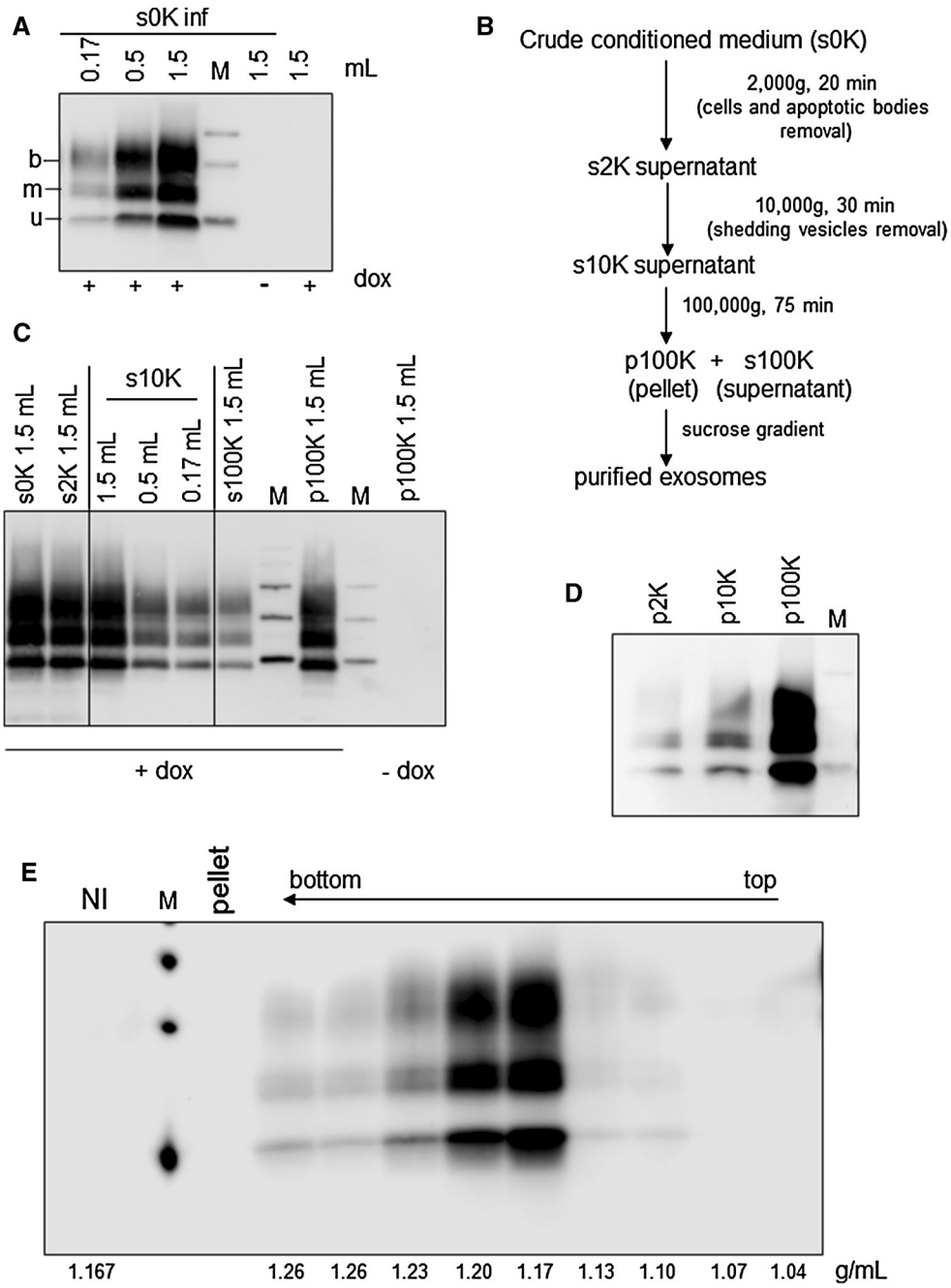
Results

Exosomal release of prions is the main pathway that leads to extracellular prions

To quantify prion infectivity released in the extracellular environment, ovRK13 cells expressing the ovine PrP^{c}

protein were mock infected or infected with the 127S ovine prion strain [19] and grown for a few weeks to ensure high levels of infection, after which conditioned media were collected. Increasing quantities of crude conditioned media (s0K) were incubated with uninfected ovRK13 cells. The inoculated cultures were maintained for 4 weeks before being analysed by immunoblotting for the presence of aggregated, PK-resistant PrP (PrP^{res}) as a marker of prion infection. As shown in Fig. 1a, ovRK13 cells inoculated with increasing quantities of s0K accumulated increasing amounts of PrP^{res}. No PrP^{res} was detected when the cell-based assay was performed in the absence of doxycycline (dox) (i.e., when ovine PrP^{c} was not expressed), demonstrating that PrP^{res} was produced de novo by the inoculated cells and did not originate from the infectious conditioned medium. Thus, released infectivity can be conveniently quantified using this cell-based assay. Crude infectious conditioned medium was then subjected to a series of centrifugation steps. This well-established procedure [26], depicted in Fig. 1b, allows the removal of potential apoptotic blebs (800–1,500 nm diameter) by a low-speed ($2{,}000\times g$) centrifugation and the elimination of shedding vesicles (up to 1,000 nm diameter) by centrifugation at $10{,}000\times g$ and leads to the eventual pelleting of the secreted exosomes in the p100K fraction after ultracentrifugation at $100{,}000\times g$. The relative infectivity of these fractions (s0K, s2K, s10K, s100K and p100K) was assessed using cell-based assay with uninfected ovRK13 as indicator cells. PrP^{res} accumulation in the cultures inoculated with the different fractions is shown in Fig. 1c. Infectivity in the crude conditioned medium (s0K) was quantitatively recovered in the supernatant after centrifugation at $2{,}000\times g$ (s2K) and $10{,}000\times g$ (s10K), indicating that infectivity associated with apoptotic bodies and large vesicles was marginal in our cell system. In contrast, ultracentrifugation of conditioned medium at $100{,}000\times g$ removed about 90 % of the infectivity (cells inoculated with 170 µL of s10K and 1,500 µL of s100K accumulated similar amounts of PrP^{res}), suggesting that most of the infectivity in s10K had been pelleted. Cell-based assays of p2K, p10K and p100K fractions confirmed these findings. Both p2K and p10K contained detectable infectivity (Fig. 1d), but most of the infectivity released by the cells was recovered in the p100K pellet (Fig. 1c, d). Finally, pelleted exosomes were further purified through floatation in sucrose gradients. Ten fractions were recovered (including the pellet of the gradient) and were tested for the presence of infectivity. Cell-based assays did not show any detectable infectivity in the gradient pellet. Instead, infectivity was detected in the sucrose fractions, indicating association with membranes. More specifically, infectivity was mainly recovered in fractions whose densities (1.169 and 1.202 g/mL) corresponded to that of exosomes (Fig. 1e) [27]. The p100K pellets harvested form uninfected and infected cultures were analysed by immunoblotting. While non-exosomal proteins (including Tom20, GM130 and Bcl2) were not detected in p100K preparations, the exosomal protein PrP^{c} was enriched in the p100K fraction (Fig. 2a). Upon infection of the cultures, PrP^{res} was detectable in the infectious p100K and enriched compared with PrP^{res} in the corresponding secreting cells (Fig. 2b). The p100K pellets from uninfected and infected ovRK13 cultures were also analysed for the exosomal proteins flotillin-1, EF1α and Alix. p100K pellets from infected and uninfected cultures had similar amounts of the three exosomal proteins (Fig. 2c), suggesting that prion multiplication does not stimulate exosome secretion.

Fig. 1 Ovine 127S prions released by ovRK13 cells are primarily associated with exosomes. **a** Cell-based assay of prion infectivity released by infected ovRK13 cells: increasing volumes (from 0.17 to 1.5 mL) of crude conditioned medium (*s0K inf*) from infected ovRK13 cultures were used to inoculate recipient uninfected ovRK13 cells. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. The dose-dependent presence of PrP^{res} indicates that s0K is infectious. No PrP^{res} was detected when the cells were inoculated with conditioned medium from non-infected cells (*last right lane*) or when recipient ovRK13 cells did not express the PrP^{c} protein (−dox). PrP^{res} is detected as unglycosylated (u), monoglycosylated (m) and biglycosylated (b) species. *M* corresponds to standard molecular mass marker proteins (20, 30 and 40 kDa). **b** Flowchart of conditioned media processing to isolate exosomes. Crude culture medium (*s0K*) was subjected to a low-speed centrifugation ($2{,}000\times g$, 20 min) to eliminate dead cells and possible apoptotic blebs. The resulting supernatant (s2K) was then centrifuged at $10{,}000\times g$ for 30 min to pellet large shedding vesicles. The s10K supernatant was collected and ultracentrifuged at $100{,}000\times g$ to separate the microvesicle fraction (pellet p100K) containing exosomes from the corresponding supernatant (s100K). **c** The indicated volumes of s0K, s2K, s10K and s100K were used to inoculate ovRK13 cells along with the p100K pellet corresponding to 1.5 mL of conditioned medium. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. The major part of the infectivity released by infected ovRK13 cells was recovered in the p100K pellet after $100{,}000\times g$ ultracentrifugation. A minor part (estimated to 10 %) remained in the s100K supernatant. No PrP^{res} was detected when the inoculated ovRK13 did not express the PrP protein (−dox). **d** Pellets corresponding to 1.5 mL of s2K, s10K and s100K supernatants (p2K, p10K and p100K, respectively) were used to inoculate ovRK13 cells. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. The major part of the infectivity released in the conditioned medium was recovered in the p100K pellet. **e** The p100K fraction isolated from an infected ovRK13 culture was fractionated by density through a discontinuous sucrose gradient. Nine fractions and the gradient pellet were collected and used to inoculate recipient ovRK13 cells for cell-based assay of infectivity. Four weeks later, the challenged cultures were tested for PrP^{res} by immunoblot. Most of the infectivity was in fractions with densities of 1.169 and 1.202 g/mL corresponding to the density of exosomes. No infectivity was detected in the gradient pellet or in a similar fraction (density = 1.167 g/mL) of a density-fractionated p100K harvested from a non-infected ovRK13 culture (NI). The density of each sucrose fraction (in g/mL) is indicated above the gel



Collectively, these findings demonstrate that exosomes are the main pathway for prion release in ovRK13 cells infected with the ovine 127S prion strain.

To generalize our findings, we performed similar experiments with RK13 cells infected with other prion strains. RK13 cells expressing the mouse PrP^{c} (moRK13) propagate the 22L mouse prion strain [20] and can be used as recipient cells to titrate 22L infectivity by the cell-based assay [18]. Conditioned medium was collected from 22L-infected moRK13 cultures and s10K supernatant was used to inoculate indicator moRK13 cells. The recipient cultures accumulated dose-dependent amounts of PrP^{res}, indicating that the conditioned medium was infectious (Fig. 3a). As shown for the 127S strain, 22L infectivity was almost exclusively found in the p100K pellet after ultracentrifugation at 100,000×*g* (Fig. 3a). After sucrose density fractionation of the p100K fraction, ten fractions were collected and tested for infectivity with moRK13 indicator cells. As was observed for the 127S strain, no infectivity was detected in the gradient pellet. The most infectious

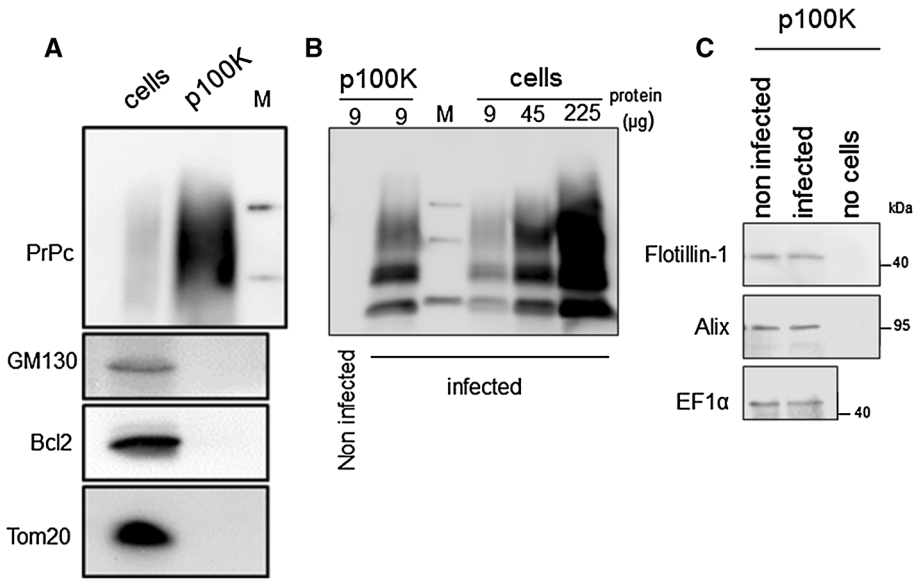


Fig. 2 Biochemical analysis of the p100K fractions. **a** The p100K fraction was isolated from uninfected ovRK13 culture medium. Equal amounts of proteins from p100K and from cell lysate of the corresponding secreting cells were analysed by immunoblotting for PrP^{c}, GM130, Bcl2 and Tom20 proteins. *M* corresponds to standard molecular mass marker proteins (30 and 40 kDa). **b** The p100K fractions (9 µg of proteins) isolated from uninfected and infected ovRK13 cultures were compared with the cell lysates of infected ovRK13 (9, 45 and 225 µg of proteins). All samples were digested with PK, and PrP^{res} was analysed by immunoblot. **c** The p100K fractions were isolated from media (20 mL) conditioned by non-infected or infected ovRK13 cells or from cell culture medium alone (no cells) and were analysed for exosome proteins (alix, flotillin-1 and EF1α) by immunoblotting. Molecular mass marker proteins in kDa are indicated on the *right*

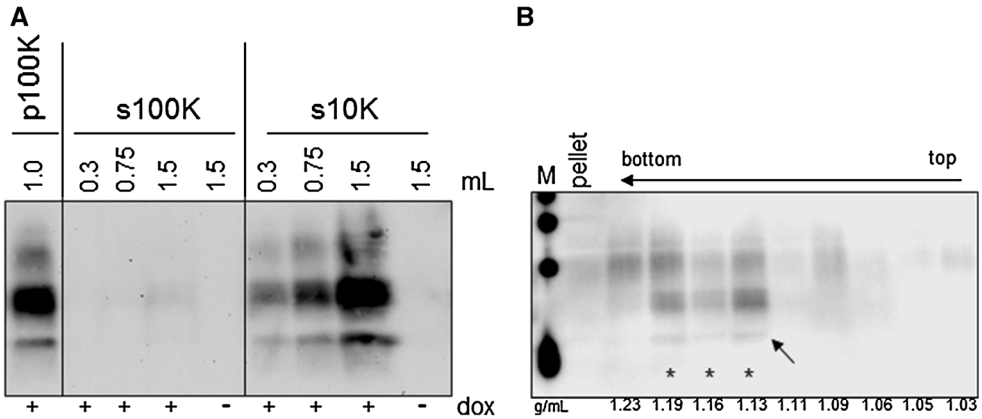


Fig. 3 22L murine prions released by moRK13 cells are primarily associated with exosomes. **a** s10K, s100K and p100K were prepared from conditioned medium of moRK13 cells infected with the 22L murine strain of prions. The indicated volumes of s10K and s100K were inoculated to moRK13 cells along with the p100K pellet corresponding to 1.0 mL of s10K. Four weeks later the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. PrP^{res} amounts in the inoculated cultures indicate that the major part of 22L infectivity released by infected moRK13 cells was recovered in the p100K pellet after 100,000×*g* ultracentrifugation. No PrP^{res} was detected with recipient cells that do not express the PrP protein (−dox). **b** The p100K fraction isolated from an infected moRK13 culture was fractionated by density through a discontinuous sucrose gradient. Nine fractions and the gradient pellet were collected and used to inoculate recipient moRK13 cells for cell-based assay of 22L infectivity. Four weeks later, the challenged cultures were tested for PrP^{res} by immunoblot. Typical PrP^{res} (the *arrow* shows the unglycosylated form of PrP^{res}) is detected in fractions labelled with a star (*asterisk*) (density 1.13–1.19 g/mL), indicating that most of the infectivity is in exosome fractions. No infectivity was detected in the gradient pellet. *M* corresponds to standard molecular mass marker proteins (20, 30 and 40 kDa). The density of each sucrose fraction (in g/mL) is indicated above the gel

fractions had densities from 1.13 to 1.19 g/mL (Fig. 3b). Therefore, the 22L strain of mouse prions is mainly secreted through the exosomal pathway.

We previously showed [20] that RK13 cells can also propagate a vole prion strain, provided they express the vole PrP^c protein (voRK13). Conditioned medium from infected voRK13 cells was prepared. Inoculation of recipient voRK13 cells with up to 1.5 mL of conditioned medium was not sufficient to lead to detectable PrP^{res} in the inoculated cells. However, the p100K fraction harvested from 12 mL of s10K supernatant was infectious for voRK13 cells (Fig. 4d), suggesting that, as was observed for ovine 127S and mouse 22L strains, the vole prion strain may also be secreted in association with exosomes.

Efficiency of prion release

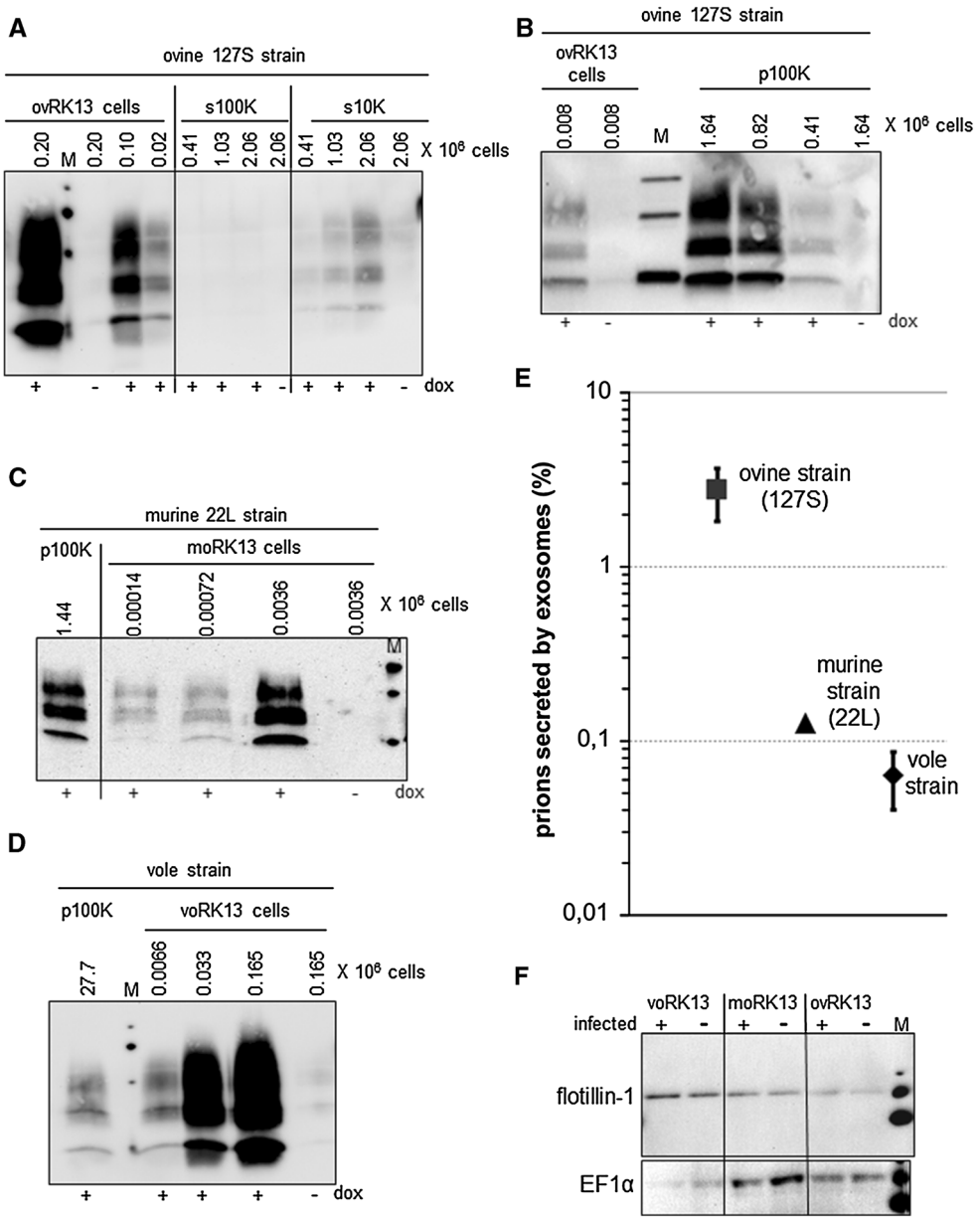
To determine the efficiency of exosomal prion secretion in each paradigm, we compared the infectivity in the p100K fraction to that in the corresponding secreting cells. Towards this end, various amounts of infected cells and of the corresponding p100K were used to inoculate healthy recipient cells, and the relative infectivity in the p100K and cell fractions was deduced from PrP^{res} accumulation in the inoculated cells (Fig. 4). Data from two experiments with ovRK13 cells infected with the ovine 127S prion strain are shown in Fig. 4a, b. Densitometric analysis of PrP^{res} in the inoculated cells indicated that infectivity in the s10K corresponded to 2.2 % of that in the corresponding secreting cells (Fig. 4a). When exosomes were pelleted by ultracentrifugation, infectivity in the resulting p100K pellet corresponded to 2 % of that in the cells (Fig. 4b). Quantification of infectivity in p100K harvested from six different infected ovRK13 cultures indicated that ~2.75 % of cellular infectivity was secreted through the exosomal pathway (Fig. 4e). When similar experiments were carried out with p100K from moRK13 infected with murine 22L prions (Fig. 4c) or from voRK13 infected with the vole prion strain (Fig. 4d), the proportion of released infectivity was much lower (0.12 and 0.06 % for the murine and vole prions, respectively, Fig. 4e). These data suggest that the ovine 127S prion strain is ~20- to 40-fold more efficiently secreted than the murine 22L and the vole prion strains. The p100K harvested from RK13 cultures infected or not with the three prion strains were analysed for flotillin-1 and EF1-α exosomal proteins. As already observed for the 127S strain (Fig. 2c), the levels of flotillin-1 and EF1-α were not higher in p100K from infected cells, providing further indication that prion infection did not stimulate exosome secretion (Fig. 4f). These data also indicate that the high efficiency of 127S ovine prions release by ovRK13 is not due to an increased secretion of flotillin-1-positive and EF1α-positive vesicles (see "Discussion").

Titration of exosomal prions

To determine the amount of infectivity in a typical exosome preparation, we harvested the p100K from 170 mL of s10K medium conditioned with ovRK13 cells infected with the 127S prion strain. This p100K was titrated by inoculating ovRK13 cells with serial ¼ dilutions. For these experiments, we performed two successive rounds of cell-based assays, as we had previously shown that two rounds increased by 100-fold the cell assay sensitivity [18]. Figure 5 shows that PrP^{res} was still detected in the cultures inoculated with p100K corresponding to 0.25 µL of conditioned medium. If one scrapie cell assay (SCA) unit is defined as the amount of infectivity that leads to a detectable infection of ovRK13 cells in our assay, an exosome preparation typically contains 0.8×10^6 SCA units per 200 mL of conditioned medium. As discussed below, the amounts of infectious exosome harvested from 127S-infected ovRK13 cells may make them valuable spiking materials to monitor prion clearance during plasma fractionation.

Discussion

Active release of prions through association with exosomes was originally described for ovRK13 and MovS cells infected with the 127S ovine prion agent [13]. Since then, studies with additional cell types (GT1-7, moRK13, N2a, B cells) and other prion strains (M1000, 22L, RML) confirmed that exosomes secreted from infected cells can carry abnormal PrP and associated infectivity [12, 14, 28, 29]. Collectively, our present work and all these findings point to the fact that exosome secretion is a general feature of the prion life cycle, at least in cultured cells. However, the fact that prions highjack the exosomal pathway does not mean that exosomes are the only route for prion release. Evidence for other pathways of prion secretion includes large vesicles budding from the plasma membrane of N2a cells [11] and association of prions with viral particles released from NIH3T3 and N2a cells [12, 30]. The relative contributions of the different routes and whether they vary depending on the secreting cell type and/or the strain of agent are open questions. In this study, we show that depletion of large shedding vesicles did not significantly decrease the infectivity of conditioned media from infected RK13 cells, indicating that budding at the plasma membrane has a minor effect on prion release by RK13 cells. In sharp contrast, infectivity was quantitatively recovered in 100,000×*g* pellets and found in the exosome-containing gradient fractions, indicating that exosomal release of prions was the main pathway that led to extracellular prions in this model. However, in the absence of experimental tools



to specifically interfere with exosome release, it is difficult to exclude that small vesicles possibly budding from the plasma membrane [31] may also be present in the exosome-containing fractions.

We believe that infectious exosomes from cultured cells may represent a pertinent, natural biological material for spiking experiments aimed at validating prion removal during the manufacturing of plasma-derived biological products. A recent study established that 1:2,000 individuals in the UK are carriers of vCJD prions in peripheral lymphoid tissues [32]. Whether these individuals will eventually develop the clinical disease during their life is unknown, but this does raise concerns regarding the possible transmission of infection, notably through blood transfusion and/or blood-derived products. Therefore, removal/inactivation of prions during plasma-manufacturing procedures and blood transfusions must be improved, in addition to continuing efforts to develop a prion blood screening test [33]. So far, the efficacy of prion removal/inactivation during these procedures has been typically evaluated with plasma exogenously spiked with either detergent-extracted prion preparations or with crude brain homogenates [34]. Because the biochemical properties of these preparations are likely to differ from those of endogenous plasma prions, there is no consensus on the clinical relevance of the data. Our suggestion to use

◀**Fig. 4** Strain-dependent efficiency of exosomal prion release. The infectivity in cells, s100K, s10K and p100K fractions from ovRK13 cultures infected with the ovine strain (**a**, **b**), moRK13 cultures infected with the murine 22L strain (**c**), and voRK13 cultures infected with the vole strain (**d**) was tested using cell-based assay. **a** Various numbers of cells and s100K and s10K harvested from the indicated numbers of cells were used to inoculate non-infected recipient ovRK13 cells. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. Quantification of the dose-dependent accumulation of PrP^{res} indicated that 2.2 % of cell infectivity was secreted in the s10K conditioned medium. *M* corresponds to standard molecular mass marker proteins (20, 30 and 40 kDa). **b** p100K fraction harvested from the indicated number of cells was used to inoculate non-infected recipient ovRK13 cells along with the cell fraction (0.008×10^6 cells). Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. Quantification of the dose-dependent accumulation of PrP^{res} indicated that 2 % of cell infectivity was recovered in the p100K. **c** The p100K fraction harvested from conditioned medium of 1.44×10^6 moRK13 cells infected with murine 22L prions was used to inoculate non-infected moRK13 cells along with the indicated number of the corresponding infected cells. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. Quantification of the dose-dependent accumulation of PrP^{res} indicated that 0.12 % of cell infectivity was recovered in the p100K. **d** The p100K fraction harvested from conditioned medium of 27.7×10^6 voRK13 cells infected with the vole prions was used to inoculate non-infected voRK13 cells along with the indicated number of the corresponding infected cells. Four weeks later, the challenged cultures were solubilized and processed for PrP^{res} detection by immunoblotting. Quantification of the dose-dependent accumulation of PrP^{res} indicated that 0.02 % of cell infectivity was recovered in the p100K. **e** The *graph* shows the proportion of infectivity recovered in the p100K fraction (in % of infectivity within the corresponding secreting cells), as determined by quantifications of PrP^{res} as shown in **a**–**d**. Data are the mean ± SEM from six different experiments (ovine 127S strain, *filled squares*) or three different experiments (murine 22L strain, *filled triangle*; vole strain, *filled diamond*). **f** p100K fractions were harvested from infected (*plus*) or mock-infected (*minus*) voRK13, moRK13 and ovRK13 cultures. p100K fractions harvested from an identical number of cells (30×10^6, corresponding to 20 mL of s10K conditioned medium) were analysed by immunoblot for flotillin-1 and EF1α exosomal proteins

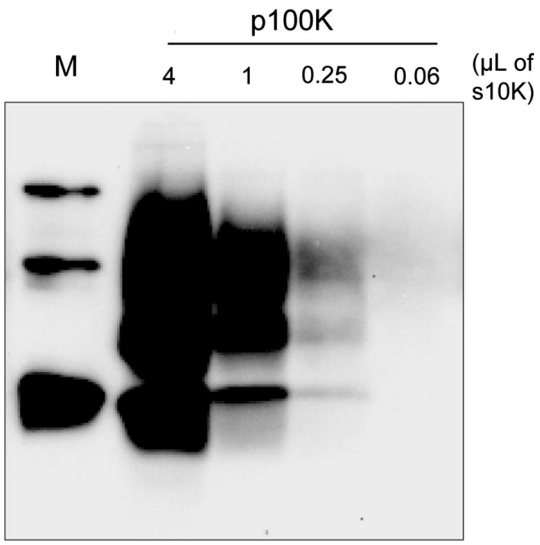


Fig. 5 Titration of infectious exosome preparation. p100K was harvested from conditioned medium (170 mL) of ovRK13 cells infected by the ovine 127S strain. The infectivity in p100K was titrated by the cell-based assay, in which recipient ovRK13 cells were inoculated with decreasing amounts of p100K (from 4 to 0.06 μL of the corresponding conditioned medium). After two rounds of cell-based assay, PrP^{res} in the inoculated cultures was analysed by immunoblotting. p100K corresponding to the equivalent of 0.25 μL of conditioned medium led to a detectable infection of the recipient cells. *M* corresponds to standard molecular mass marker proteins (20, 30 and 40 kDa)

infectious exosomes as spiking material is based on several reasons. First, these biological vesicles have a relatively defined size and might be present endogenously in contaminated plasma. Second, their isolation from ovRK13-conditioned media is straightforward (3 centrifugation steps). Finally, ovRK13 cells persistently infected with 127S secrete substantial amounts of exosome-associated infectivity, close to 10^6 SCA infectious units per 200 mL of culture medium. This titre is high enough to control prion removal/inactivation of spiked plasma over several log10 units.

While prion secretion appears to be a general feature of prion metabolism in infected cultured cells, evidence for infectious exosomes in animals and for their possible contribution to TSE pathogenesis is still lacking. Because EV release and subsequent uptake are very dynamic processes, it may be very difficult to provide direct ultrastructural evidence for the presence of EV in infected solid tissues [35]. Alternatively, several infectious body fluids, including urine, milk [36] and blood [37–40], contain exosomes [41–43]. Consistent with a possible contribution of EV and/or exosomes to blood prions, significant infectivity is detected in the plasma of infected rodent [44–46] and sheep [37, 47, 48]. Based on our data with 127S-infected cultured cells, the plasma of sheep infected with the 127S ovine agent [47] might be a favourable biological material for the in vivo detection of infectious exosomes. In various diseases, notably cancer [49], exosomes from body fluids are promising tools for non-invasive diagnostics. The recent finding that exosomes released from prion-infected cells have a distinct miRNA signature [50] raises the possibility that exosomes may be valuable targets for antemortem TSE diagnosis as well.

Thanks to the development of cell-based assays for prion detection [18, 51], we show that distinct prion strains may promote their secretion through the exosomal pathway with different efficiencies, with ovine 127S prions being 20- to 40-fold more efficiently secreted than the murine 22L and the vole strains. Although we did not quantify the released vesicles, we detected similar amounts of exosome

proteins in media from infected and non-infected cells, suggesting that prion multiplication does not stimulate exosomal release. This implies that prions are incorporated into constitutively secreted exosomes. An understanding of why ovine 127S prions are much more efficiently targeted to exosomes than mouse 22L or bank vole prions will require further studies. The biogenesis of exosomes and how cargoes are targeted into these vesicles are still at an early stage of understanding [52]. Secreted exosomes correspond to intraluminal vesicles (ILVs) [53] formed by inward budding of the limiting membrane of endosomes [54]. A subset of these multivesicular endosomes (MVEs) can fuse with the plasma membrane, and the released ILVs are named exosomes [55, 56]. The endosomal sorting complex required for transport (ESCRT) machinery comprised five distinct protein complexes and an array of accessory factors, which together are involved in cargo recognition and sequestration in particular endosome membrane domains followed by inward membrane invagination and subsequent scission to generate ILVs in the lumen of MVEs [57]. Even though ESCRT is required for MVE biogenesis in yeast, the situation is far more complex in mammalian cells, where MVE biogenesis can still be observed upon ESCRT inhibition [58]. Accordingly, other ESCRT-independent mechanisms have recently been described for the sorting of some cargoes towards exosomes [59–61]. It is currently unclear to what extent these different mechanisms may be cell type specific and/or cargo specific [52]. More specifically, the mechanism(s) by which PrP^{c} and PrP^{sc} are directed to the exosome secretion pathway is currently under investigation. Using pharmacological and lentivirus-mediated knockdown approaches, we have generated results indicating that 127S prions are secreted through both ESCRT-dependent and -independent pathways (submitted), and it will be of interest to determine whether the same pathways contribute to mouse 22L and vole prions release. However, an interesting possibility would be that some structural features of abnormal 127S PrP make it more prone to exosome incorporation. It is now increasingly recognized that abnormal PrP is in fact a population of diverse entities with distinct biochemical features and possibly different biological properties [62]. Regarding 127S prions, we provided evidence that a portion of infectious PrP^{res} aggregates have a small size [63]. In independent studies, velocity and equilibrium sedimentations were used to separate a distinct subset of highly infectious small PrP^{res} aggregates from the bulk of highly aggregated 127S PrP^{res} [64, 65]. An appealing possibility would be that, due to their 50- to 100-nm diameter, exosomes preferentially incorporate small 127S PrP^{res} aggregates. Fractionation and/or sedimentation of exosome-associated 127S PrP^{res}, coupled with infectivity detection by the ovRK13 cell-based assay, will be required to explore this hypothesis.

Acknowledgments We thank Dr. V. Setola for critical reading of the manuscript. This work was partly supported by a grant from ANR-08-MIEN-010 (France) and Z. E. Arellano-Anaya was a recipient of a fellowship from Mexican CONACYT. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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