

# Recovery of Small Infectious PrP<sup>res</sup> Aggregates from Prion-infected Cultured Cells\*

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Prion diseases are characterized by deposits of abnormal conformers of the PrP protein. Although large aggregates of proteinase K-resistant PrP (PrP<sup>res</sup>) are infectious, the precise relationships between aggregation state and infectivity remain to be established. In this study, we have fractionated detergent lysates from prion-infected cultured cells by differential ultracentrifugation and ultrafiltration and have characterized a previously unnoticed PrP species. This abnormal form is resistant to proteinase K digestion but, in contrast to typical aggregated PrP<sup>res</sup>, remains in the soluble fraction at intermediate centrifugal forces and is not retained by filters of 300-kDa cutoff. Cell-based assay and inoculation to animals demonstrate that these entities are infectious. The finding that cell-derived small infectious PrP<sup>res</sup> aggregates can be recovered in the absence of strong *in vitro* denaturing treatments now gives a biological basis for investigating the role of small PrP aggregates in the pathogenicity and/or the multiplication cycle of prions.

Prion diseases are caused by the misfolding of the endogenously expressed PrP protein, and the most widely accepted model of prion multiplication is that abnormally folded PrP recruits and converts the host PrP into new abnormal forms (1–3). Historically, abnormal PrP was operationally defined as insoluble and resistant to protease digestion (4) and was subsequently termed PrP<sup>res</sup>.<sup>4</sup> Although normal PrP protein is soluble in detergents and sensitive to proteinase K (PK) digestion, part of PrP in infected tissues is PK-resistant and recovered in the pellets of centrifuged samples. These large PrP<sup>res</sup> aggregates are infectious (5), but it is unclear whether infectivity is entirely congruent with these species (6, 7) or whether they are the more efficient species to promote infection. Efficient transmission in the absence of detectable typical PrP<sup>res</sup> has been observed in some models of prion diseases (8, 9), and in other situations, infectivity and the bulk of large PrP<sup>res</sup> aggregates have distinct sedimentation profiles in density gradients (10). In addition, large aggregates of PrP<sup>res</sup> are not the only abnormal forms in

diseased tissues, as exemplified by the characterization of PK-sensitive abnormal forms of PrP (11–15). Collectively, these and other findings (16) indicated that abnormal PrP is a set of diverse populations, displaying various biochemical properties and different morphological deposits in the brain, and their biological properties have yet to be established.

Here, we investigated the diversity of infectious abnormal PrP species in a cell line and in neuronal primary cultures infected with a sheep scrapie agent. Although we obtained no evidence for the presence of PK-sensitive infectivity, we identified a previously unnoticed abnormal PrP species. Solubility and ultrafiltration assays indicate that these PK-resistant PrP entities are small aggregates. These species are infectious and may explain the perplexing presence of infectivity in brain samples devoid of detectable large aggregates of PrP<sup>res</sup>.

## EXPERIMENTAL PROCEDURES

**Cells and Transgenic Mice**—Rov cells (the Rov9 clone) were maintained at 37 °C in 5% CO<sub>2</sub> in Opti-MEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 10 µg/ml streptomycin (17). To induce the expression of ovine PrP, 1 µg/ml doxycycline (Sigma-Aldrich) was added to the culture medium.

Primary neuronal cultures were established from transgenic mice expressing the ovine PrP (*tg338*) or the human PrP on a mouse PrP<sup>0/0</sup> background, as described (18). Cerebellar granule neurons (CGNs) were extracted from 5- to 7-day-old mice by enzymatic and mechanical dissociation and were plated at a density of 2,500 cells/mm<sup>2</sup> in plastic culture wells coated with 10 µg/ml poly-D-lysine (Sigma-Aldrich) at 37 °C and 5% CO<sub>2</sub>. Cells were cultured in DMEM-GlutaMAX (Invitrogen) containing 10% heat-inactivated fetal bovine serum, 25 mM KCl, penicillin and streptomycin, N2 and B27 supplements (Invitrogen). The medium was complemented weekly with 1 mg/ml glucose and 10 µM antimetabolites uridine and fluorodeoxyuridine (Sigma-Aldrich). For immunofluorescence analysis, CGNs were plated on poly-D-lysine-coated glass coverslips and analyzed 13–20 days later. Cultures were prefixed by adding 1 volume of paraformaldehyde (4% w/v in PBS) to the culture medium. Ten minutes later, the cells were subjected to a second round of fixation by adding 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.2% Triton X-100 in PBS and stained for microtubule-associated protein 2 with mAb HM-2 (Sigma-Aldrich) and for β-tubulin III with affinity-purified polyclonal antibodies (Sigma-Aldrich). Bound antibodies were visualized with Alexa Fluor-conjugated secondary anti-

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<sup>4</sup> The abbreviations used are: PrP<sup>res</sup>, proteinase-resistant PrP protein; CGN, cerebellar granule neuron; PK, proteinase K.

## Small Infectious PrP<sup>res</sup> Aggregates

bodies (Molecular Probe), and coverslips were mounted on slides using ProLong Gold antifade reagent (Invitrogen) containing DAPI.

Ovine transgenic mice (*tg338* line) are transgenic for the ovine PrP on a mouse PrP<sup>0/0</sup> background (19). The mouse line transgenic for the human PrP will be described elsewhere.

**Prion Agent and Infection of Cultured Cells**—The sheep isolate (PG127) (20) propagated once in sheep was used to infect *tg338* mice. At the terminal stage, brains were homogenized at 10% (w/v) in 5% sterile glucose using a high speed homogenizer (TeSeE Precess 48 system), and infectious inoculum was heated at 70 °C for 20 min. Persistently infected Rov cultures were obtained by exposing uninfected cells to 0.5% infectious brain homogenate for a few days. The resulting cultures were grown for 1–2 months and used to prepare cell lysates. Mock-infected Rov cultures were grown and processed in parallel. CGNs (at day 3 after plating) were infected by diluting the 10% infectious homogenate 1,000-fold in cell culture medium.

**PK Digestion of Cell Lysates and Brain Extracts**—Cell cultures were rinsed with cold PBS and solubilized for 10 min at 4 °C in Triton-DOC lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, 0.5% sodium deoxycholate). The lysates were clarified by low speed centrifugation (425 × *g*, 1 min), and cellular proteins in the postnuclear supernatants were quantified by bicinchoninic acid (Pierce). Digestion with PK (recombinant grade; Roche Applied Science) was performed for 2 h at 37 °C with a mass ratio of 4 μg of PK/mg of cellular proteins, and the reaction was stopped by the addition of Pefabloc (Sigma-Aldrich) to 4 mM. Small volumes of 10% brain homogenates were solubilized in Triton-DOC lysis buffer and processed as cell lysates for detection of PrP<sup>res</sup>. PK-digested samples were centrifuged for 1 h at 10,000–20,000 × *g*, and pellets were analyzed by Western blotting.

**Search for PK-sensitive Infectivity in Cell Lysates**—Lysates (the equivalent of 250 μg of proteins) from infected Rov cultures were incubated for 2 h at 37 °C with 1 μg of PK, and digestion was stopped with Pefabloc. Mock-treated lysates were incubated with Pefabloc and no PK. Proteins were precipitated with 4 volumes of methanol, resuspended in PBS, and used to inoculate Rov cells.

**Isolation of Soluble and Insoluble Fractions**—Preparative isolation of the soluble and insoluble fractions was carried out with three different infected Rov cultures with similar results. Typically, 35 ml of PK-digested cell lysates (1 mg/ml) were centrifuged at 100,000 × *g* ( $g_{av}$ ) for 1 h at 4 °C in polyallomer tubes in a SW32 rotor (Beckman). The supernatant was removed, and 4 volumes of methanol were added to precipitate proteins. The insoluble pellet was dried at room temperature, transferred with 400 μl of PBS into a microtube, and stored at –20 °C. The methanol-precipitated supernatant fraction was centrifuged for 30 min at 10,000 × *g* in 50-ml tubes, and pelleted material was air-dried and resuspended in 400 μl of PBS and transferred into a microtube. Pellets from both fractions were sonicated for a total of 6 min at power 9 with a Cup-Horn apparatus to help dissolution and stored at –20 °C until used for immunoblotting, guanidine denaturation assays, cell-based assay, and bioassay in mice. In some experiments, the supernatant obtained after a 1-h centrifugation at 100,000 × *g* was transferred to a

new clean tube and subjected to a second centrifugation at 100,000 × *g*.

In some experiments, analytical PK-digested samples were centrifuged in 1.5-ml microtubes either in a TLA-110 rotor (Beckman) or in a microcentrifuge. Rotors with different sedimentation pathlengths have distinct efficiencies to pellet a given particle, and these can be compared using their *k* factors. At 100,000 × *g*, *k* factors of SW32 and TLA-110 rotors are 262 and 50, respectively, indicating that the TLA-110 is about 5-fold more efficient than SW32 to pellet a given particle. Therefore, centrifugation at 20,000 × *g* or 100,000 × *g* with TLA-110 corresponds to 100,000 × *g* or 500,000 × *g*, respectively, with the SW32 rotor. Pellets and methanol-precipitated supernatants were resuspended in small volumes of PBS without sonication and analyzed by immunoblotting and in the cell-based assay.

**Guanidine Denaturation Assays**—Insoluble and soluble fractions in PBS were mixed with a noninfected cell lysate to achieve a final protein concentration of 870 μg/ml. Aliquots (30 μl) were mixed with the same volume of H<sub>2</sub>O or GdnHCl to achieve GdnHCl final concentrations of 1, 1.5, and 2 M and were incubated for 1 h at room temperature. After methanol precipitation, the samples were digested with 0.1 μg of PK for 2 h at 37 °C and analyzed by immunoblotting.

**Immunoblotting**—Samples were separated by 12% SDS-PAGE before transfer to PVDF membranes (Bio-Rad). The Western blots were stained for PrP with Sha31 mAb (21). Filters were developed using an ECL Plus reagent kit (Amersham Biosciences-GE Healthcare) and visualized with a Bio-Rad Versa-Doc imaging system. The signals were quantified with Quantity One software (Bio-Rad).

**Ultrafiltration of Cell Lysates**—The total and the soluble fractions of PK-digested infected Rov cell lysates were ultrafiltered as follows. We used filters with cutoffs of 0.2 μm, 1,000 kDa, 300 kDa (Vivaspin 500; Sartorius), and 100-kDa (Microcon; Millipore). Before use, the filters were rinsed with the lysis buffer to remove the preservatives. For each filter, 350 μg of PK-digested lysates (1 mg/ml) were loaded. The ultrafiltration was performed at 5,000 rpm, at room temperature for 15 min in a microcentrifuge. The volumes of the collected filtrates were the same as the loaded volumes. The retentates were recovered by the addition of 350 μl of the lysis buffer. The filtrates and retentates were either methanol-precipitated or subjected to centrifugation (10,000 × *g*, 1 h) to generate soluble and insoluble fractions.

**Immunoprecipitation of PrP<sup>res</sup>**—Purified βS36 antibody (22) and BSA were coupled to Dynabeads M-270 (Invitrogen) following the manufacturer's protocol. Lysate from infected Rov cells (1 mg/ml) was PK-digested and centrifuged for 1 h at 100,000 × *g* in the SW32 rotor. Supernatant (1.1 ml) was incubated with 5.5 mg of βS36- or BSA-coupled Dynabeads for 2 h, at room temperature. A fraction of the supernatant (100 μl) and the equivalent amount of magnetic beads were analyzed by Western blotting. The remainder (1 ml) was methanol-precipitated, resuspended with sonication in 300 μl of PBS, and used in the cell-based assay.

**Cell-based Assay and Bioassay of the Soluble and Insoluble Fractions**—Uninfected Rov cells were seeded in 6-well plates in the presence of doxycycline. When confluent, the cultures were

exposed to 3 ml of culture medium containing 2, 8, or 32  $\mu$ l of the insoluble or soluble preparative fractions. In parallel, 32  $\mu$ l of each fraction was used to inoculate Rov cells in the absence of doxycycline. For cell-based assay of immunodepleted supernatants, from 30 to 300  $\mu$ l were tested. Infection was allowed to proceed for 3–4 weeks at the end of which the cultures were solubilized. In each assay, 3 ml of culture medium containing serial 10-fold dilutions of the PG127 10% infectious brain homogenate was inoculated in parallel to Rov cells. Cell lysates (500  $\mu$ g of proteins) were PK-digested and centrifuged at  $10,000 \times g$  for 1 h, and pellets were analyzed for insoluble PrP<sup>res</sup> by Western blotting.

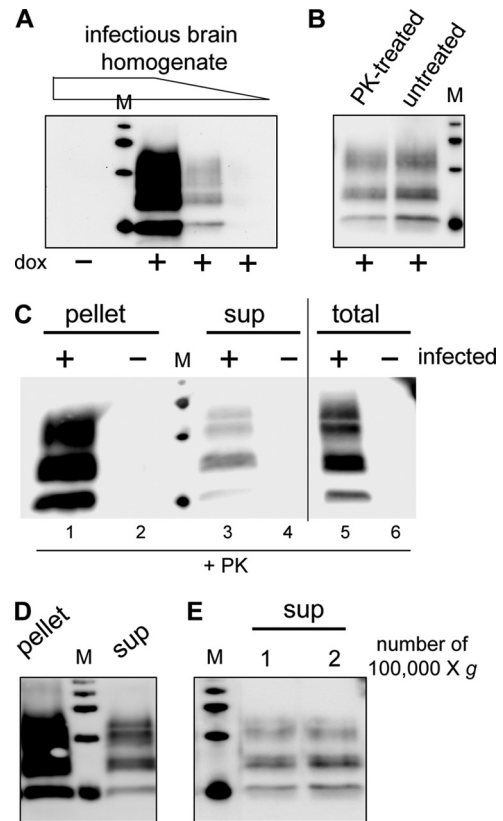
Insoluble and soluble preparative fractions (4  $\mu$ l each, corresponding to 350  $\mu$ g of cellular proteins digested with PK) were diluted to 200  $\mu$ l with 10% brain homogenate from a healthy sheep, and 20  $\mu$ l was inoculated intracerebrally into six *tg338* mice. Mice were then clinically monitored until the occurrence of scrapie clinical signs, at which time they were euthanized.

## RESULTS

**Detection of Infectious PrP<sup>res</sup> in the  $100,000 \times g$  Supernatants of PK-digested Cell Lysates**—The sheep prion agent used here was selected because it multiplies stably, efficiently, and apparently faithfully in previously described Rov and MovS cell lines (23) and in cultured primary neurons (18). In addition, infectivity levels can be quantified either through bioassay in *tg338* transgenic mice expressing the ovine PrP protein (20) or through a cell-based assay.

Previous studies reported the presence of abnormal, yet PK-sensitive, forms of PrP in infected tissues and cultured cells from various species (11–15). However, it is not known whether these particular abnormal PrPs are infectious. To clarify this issue in our model, chronically infected Rov cultures were solubilized in nondenaturing detergents. Cell lysates were subjected to PK digestion (2 h at 37 °C, with 4  $\mu$ g of PK/mg of cellular proteins) or left untreated, and the samples were precipitated with methanol. Infectivity in the resuspended materials was quantified through a Rov cell-based assay. In this assay, Rov cultures inoculated with increasing infectivity accumulate dose-dependent amounts of PrP<sup>res</sup> (Fig. 1A). Uninfected Rov cells were exposed to mock- and PK-digested infected Rov extracts, and the inoculated cultures were analyzed 3 weeks later for the presence of PrP<sup>res</sup>. We found that Rov cells inoculated with mock- or PK-digested samples had accumulated similar amounts of PrP<sup>res</sup>, indicating that infectivity in the two inocula was similar (PrP<sup>res</sup> amounts in cultures inoculated with mock-treated lysates was  $19.5\% \pm 22\%$  (mean  $\pm$  S.E.,  $n = 6$ ) above those in cultures inoculated with PK-treated lysates; see a representative Western blot analysis in Fig. 1B). We thus concluded that the vast majority of infectivity propagated in Rov cells is resistant to PK digestion and that PK-sensitive infectivity, if any, is marginal.

Fig. 1C illustrates the PrP<sup>res</sup> species recovered in nondenaturing detergent lysates of chronically infected Rov cells. Immunoblot analysis of methanol-precipitated, PK-digested samples reveals the presence of PrP<sup>res</sup> in lysates from infected cultures (lane 5). We reasoned that small PrP<sup>res</sup> aggregates, if present, might remain in the supernatant after centrifugation of



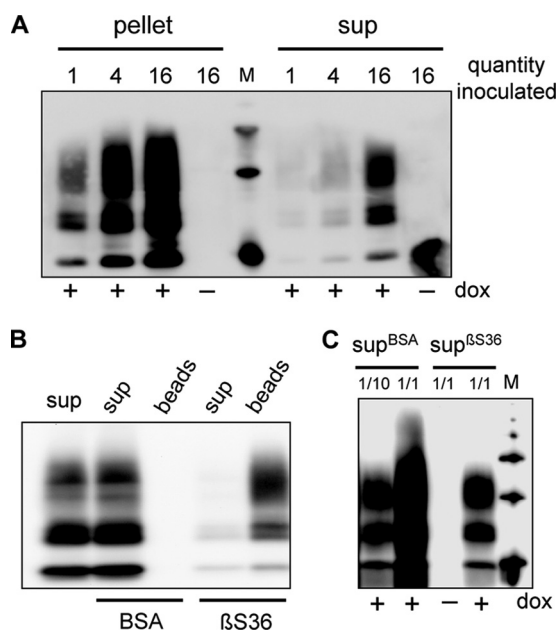
**FIGURE 1. PrP<sup>res</sup> species is present in the soluble  $100,000 \times g$  fraction of infected Rov cells.** A, Rov cell-based assay is shown. Uninfected Rov cells were inoculated with three 10-fold dilutions of an infectious brain homogenate. Three weeks later, the inoculated cultures were detergent-extracted and digested with PK, and PrP<sup>res</sup> was analyzed by immunoblotting. Please note that no PrP<sup>res</sup> was detected when inoculated Rov cultures did not express ovine PrP (*dox*–) and that the amounts of accumulated PrP<sup>res</sup> are dependent on the amount of infectivity used to inoculate the cells. *M* are standard molecular mass marker proteins (20, 30, 40, and 50 kDa). B, cell-based assay indicates that infectivity propagated in Rov cells is resistant to PK digestion. Infectious Rov cell extracts either PK-treated or left untreated were used to inoculate uninfected Rov cells. Three weeks later, accumulated PrP<sup>res</sup> in the inoculated cultures was analyzed by immunoblotting. C, lysates (250  $\mu$ g of proteins) from mock-infected (–) or infected (+) Rov cultures were digested with PK and either methanol-precipitated (lanes 5 and 6) or centrifuged at  $10,000 \times g$  for 1 h to obtain pellet and supernatant (*sup*) fractions (lanes 1–4). All the samples were analyzed for the presence of PrP<sup>res</sup> by immunoblotting. D, PK-digested lysates from infected Rov cells were centrifuged for 1 h at  $100,000 \times g$  (SW32 rotor). Equivalent amounts of the pellet or the supernatant (*sup*) fractions were analyzed for PrP<sup>res</sup>. E, PrP<sup>res</sup> in the supernatant fractions after one and two successive centrifugations at  $100,000 \times g$  was analyzed. 71% of PrP<sup>res</sup> remained in the supernatant after a second round of ultracentrifugation ( $n = 2$ ).

PK-digested lysates. PK-digested lysates were subjected to a standard centrifugation step (e.g.  $10,000$ – $20,000 \times g$  for 1 h in a microcentrifuge). Western blot analysis of pellets revealed the presence of PrP<sup>res</sup> (lane 1), consistent with the known insolubility of large PrP<sup>res</sup> aggregates. Strikingly, a PrP<sup>res</sup> signal was consistently observed when material from methanol-precipitated supernatant was analyzed (lane 3). No PrP<sup>res</sup> was observed in either fraction when uninfected cultures were analyzed (lanes 2, 4, and 6).

To characterize the PrP<sup>res</sup> species in the supernatant and pellet fractions, large volumes of infected cell lysates were digested with PK and ultracentrifuged at  $100,000 \times g$  for 1 h in a SW32 rotor to generate soluble (supernatant) and insoluble (pellet) fractions. Soluble material was recovered by methanol

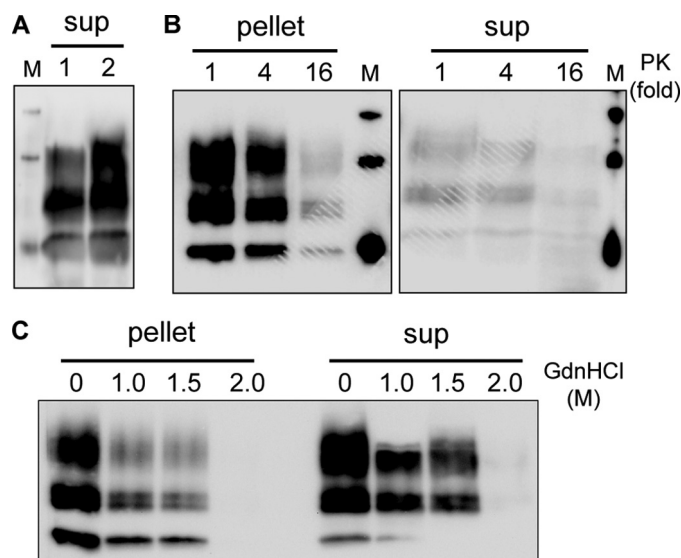


## Small Infectious PrP<sup>res</sup> Aggregates



**FIGURE 2. PrP<sup>res</sup> from the soluble fraction is infectious in the Rov cell-based assay.** A, pellet and supernatant (*sup*) fractions were recovered after  $100,000 \times g$  centrifugation of PK-digested lysates from infected Rov cells. Equivalent portions (from 1 to 16, 1 corresponds to  $175 \mu\text{g}$  of proteins in the original lysate) of each fraction were used to infect target Rov cultures. Three weeks later the cultures were solubilized and analyzed for the presence of PrP<sup>res</sup>. No PrP<sup>res</sup> was detected when inoculated Rov cultures did not express ovine PrP (*dox*<sup>-</sup>). B, PrP<sup>res</sup> was depleted from the supernatant fraction. PrP<sup>res</sup> remaining in the supernatant fraction (*sup*) after immunoprecipitation with  $\beta\text{S36}$  mAb- or BSA-coupled magnetic beads was analyzed. Immunoprecipitated PrP<sup>res</sup> was also analyzed in parallel (*beads*). The anti-PrP mAb used to reveal the blot was coupled with peroxidase. C, Rov cells were infected with immunodepleted supernatants. Various amounts (from 1/10 to 1/1) of either  $\beta\text{S36}$ -depleted supernatants (*sup* <sup>$\beta\text{S36}$</sup> ) or control BSA-depleted supernatants (*sup* <sup>$\text{BSA}$</sup> ) were used to infect target Rov cells. Four weeks later, the cultures were solubilized and analyzed for the presence of PrP<sup>res</sup>. No PrP<sup>res</sup> was detected when inoculated Rov cultures did not express ovine PrP (*dox*<sup>-</sup>).

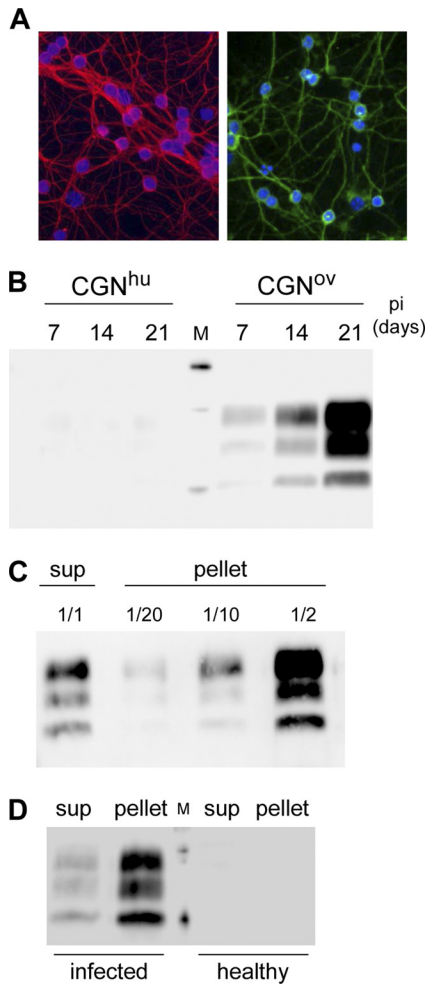
precipitation and resuspended in PBS by sonication along with the original insoluble pellet. Immunoblot analysis of the supernatant and pellet fractions showed that PrP<sup>res</sup> was still present in  $100,000 \times g$  supernatants (Fig. 1D). Importantly, this PrP<sup>res</sup> species quantitatively remained in the soluble fraction when the supernatant was submitted to a second  $100,000 \times g$  centrifugation step (Fig. 1E). Quantification through Western blot analysis of various amounts of the two fractions indicated that PrP<sup>res</sup> in the supernatant represents  $12\% \pm 8\%$  of total PrP<sup>res</sup> (mean  $\pm$  S.D., four determinations from three different infected cultures). To test for the presence of infectivity in the soluble fraction, we first used the Rov cell-based assay. Uninfected Rov cells were exposed to increasing equivalent amounts of the soluble and insoluble fractions and analyzed 3 weeks later for the presence of PrP<sup>res</sup>. Although no PrP<sup>res</sup> was observed in control cells not expressing ovine PrP, the dose-dependent presence of PrP<sup>res</sup> in Rov cells challenged with the  $100,000 \times g$  supernatant demonstrated that the supernatant was infectious (Fig. 2A). After quantification of PrP<sup>res</sup> levels in the cultures exposed to the different amounts of the soluble and insoluble fractions, we estimated that  $18.5\% \pm 5\%$  of infectivity was soluble (mean  $\pm$  S.D., four determinations from three experiments). To investigate the physical support for infectivity, we sought to deplete PrP<sup>res</sup> from the infectious supernatants by immunoprecipita-



**FIGURE 3. PrP<sup>res</sup> from supernatant is not generated through PK digestion of insoluble PrP<sup>res</sup>, and both species have similar resistance to PK digestion and GdnHCl denaturation.** A, supernatant fractions were recovered after a 1-h centrifugation at  $10,000 \times g$  before (*lane 1*) or after (*lane 2*) PK digestion of cell lysates. Supernatant from the undigested lysate was PK-digested, and both samples were analyzed for PrP<sup>res</sup> by Western blotting. B, cell lysates were digested with increasing amounts of PK (1 stands for  $1 \mu\text{g}$  of PK/ $250 \mu\text{g}$  of cellular proteins) and centrifuged at  $10,000 \times g$  for 1 h. Pellets and supernatants (*sup*) were tested for PrP<sup>res</sup> by Western blotting. C, pellet and supernatant (*sup*) fractions were incubated in the presence of the indicated concentrations of GdnHCl for 1 h. The samples were then digested with PK and analyzed for PrP<sup>res</sup> by Western blotting.

tion with  $\beta\text{S36}$ , a monoclonal antibody that specifically immunoprecipitates PrP<sup>res</sup> aggregates (22).  $\beta\text{S36}$ - but not BSA-coupled magnetic beads removed most (85%,  $n = 2$ ) of the PrP<sup>res</sup> in the  $100,000 \times g$  supernatants (Fig. 2B). Strikingly,  $\sim 90\%$  ( $n = 2$ ) of infectivity was also removed from the immunodepleted supernatants, as assessed by Rov cell-based assay (Fig. 2C). Soluble and insoluble fractions were also inoculated into *tg338* transgenic mice highly susceptible to the ovine prion strain used in this study (19). All of the inoculated mice died with typical neurological symptoms and accumulated abnormal PrP in their brains (data not shown). Mice inoculated with the pellet and supernatant fractions died 74 days  $\pm$  4 days ( $n = 5$ ) and 73 days  $\pm$  4 days ( $n = 4$ ) after inoculation, respectively. Mice inoculated with fractions from uninfected cultures remained clinically healthy and were killed at 110 days. These similar incubation periods correspond to infectious titers within the same order of magnitude and confirm that the  $100,000 \times g$  supernatants are infectious. Altogether, our data show that PK-resistant PrP recovered in a detergent-soluble fraction is infectious.

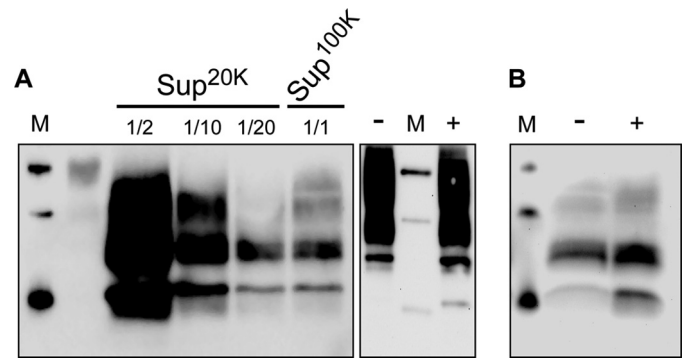
**PrP<sup>res</sup> Is Recovered in the Detergent-soluble Fraction of Infected Neurons and Prior to PK Digestion**—We then asked whether PrP<sup>res</sup> in the soluble fraction could be a by-product generated during PK digestion of insoluble PrP<sup>res</sup>. To explore this possibility, analytical volumes ( $<1$  ml) of lysates were centrifuged before PK digestion. The soluble fractions were then PK-digested and analyzed by Western blotting. Fig. 3A shows that the PrP<sup>res</sup> species can also be detected in the supernatants of non-PK-digested cell lysates, indicating that PK digestion is not required to generate this PrP<sup>res</sup> species. To examine whether PrP<sup>res</sup> species in the soluble and insoluble fractions



**FIGURE 4. PrP<sup>res</sup> is present in detergent-soluble fractions from infected cultures of CGNs and from infected brain tissue.** *A*, fluorescence analysis of *tg338*-derived cerebellar granule neurons (CGN<sup>hu</sup>) stained for microtubule-associated protein 2 (green, right) and  $\beta$ -tubulin III (red, left) after 13 and 20 days *in vitro*, respectively. Nuclei are in blue. *B*, kinetics of PrP<sup>res</sup> accumulation in infected CGN<sup>ov</sup> cultures. Proteins (75  $\mu$ g) from cell lysates obtained at 7, 14, and 21 days postinfection (*pi*) were PK-digested and centrifuged at  $10,000 \times g$  for 1 h. The pellets were analyzed to detect PrP<sup>res</sup>. *C*, detection of PrP<sup>res</sup> in the soluble fraction of PK-digested CGN<sup>ov</sup> lysates. Proteins (75  $\mu$ g) from infected CGN<sup>ov</sup> cultures (21 days postinfection) were PK-digested and centrifuged at  $10,000 \times g$  for 1 h. PrP<sup>res</sup> in the supernatant (*sup*) and in various portions of the pellet were analyzed by Western blotting. *D*, brain tissues from terminally sick or healthy *tg338* mice. Tissues were solubilized, and 50  $\mu$ g of brain proteins were PK-digested and centrifuged at  $10,000 \times g$  for 1 h. PrP<sup>res</sup> in the pellet and the supernatant (*sup*) fractions was analyzed.

differ in their PK resistance, cell lysates from infected cells were digested with increasing amounts of PK before isolation of the soluble and insoluble fractions. Immunoblot analysis (Fig. 3*B*) revealed that PrP<sup>res</sup> in the supernatant is as PK-resistant as insoluble PrP<sup>res</sup>. To study the structural stability of PrP<sup>res</sup> in the soluble and insoluble fractions, we performed guanidine denaturation assays. Both fractions were incubated with increasing amounts of GdnHCl and then digested with PK. Immunoblot analysis indicated that PrP<sup>res</sup> species in the soluble and insoluble fractions have similar stabilities to denaturation by GdnHCl (Fig. 3*C*).

The ovine prion agent used here can also be propagated in *tg338*-derived neuronal primary cultures (18). Cultured CGNs from either *tg338* (CGN<sup>ov</sup>) (Fig. 4*A*) or transgenic mice express-



**FIGURE 5. PrP<sup>res</sup> from supernatants is not fully soluble.** *A*, left, PK-digested lysates from infected Rov cells were centrifuged in a TLA-110 rotor at 20,000 or  $100,000 \times g$  for 1 h. Various portions of the corresponding supernatants (*Sup*<sup>20K</sup> or *Sup*<sup>100K</sup>, respectively) were analyzed for PrP<sup>res</sup> by Western blotting. Right, undigested lysates from uninfected Rov cells were analyzed for normal PrP before (–) or after (+)  $100,000 \times g$  centrifugation. Please note that these ultracentrifugation conditions correspond to  $100,000 \times g$  or  $500,000 \times g$  with the SW32 rotor used previously (see “Experimental Procedures”). *B*, soluble fraction of a PK-digested cell lysate was ultracentrifuged for 1 h at  $200,000 \times g$  in a TLA-110 rotor either directly (–) or through a 10% (w/v) sucrose cushion (+). The pellets were analyzed for PrP<sup>res</sup> by Western blotting.

ing a heterologous PrP (human PrP, CGN<sup>hu</sup>, used as nonpermissive, negative controls) were infected 3 days after plating. The cells were solubilized and analyzed 7, 14, or 21 days after infection. A marked, time-dependent accumulation of PrP<sup>res</sup> was observed in CGN<sup>ov</sup>, demonstrating active prion multiplication in the primary neuronal cultures derived from *tg338* mice (Fig. 4*B*). Analysis of supernatant and pellet fractions from PK-digested CGN<sup>ov</sup> lysates revealed that  $12.5\% \pm 4\%$  of the total PrP<sup>res</sup> was in the soluble fraction (mean  $\pm$  S.D. of five determinations from two different infected CGN<sup>ov</sup> cultures) (Fig. 4*C*). Lastly, brain homogenates from *tg338* mice, either healthy or at the terminal stage, were solubilized in the Triton-DOC lysis buffer, subjected to PK digestion, and centrifuged. Analysis of the supernatant and pellet fractions showed that PrP<sup>res</sup> was also present in the soluble fraction of infected brain tissue (Fig. 4*D*).

**Infectious PrP<sup>res</sup> in the Detergent-soluble Fraction Are Small Aggregates**—As a first step to investigate the reasons for apparent solubility of PrP<sup>res</sup> (small size or low density), we tested whether PrP<sup>res</sup> in the soluble fraction would be pelleted if centrifuged at a higher speed. To apply higher centrifugation forces, we used a TLA-110 rotor (Beckmann). Because its *k* factor is about 5-fold less than that of SW32, TLA-110 is about 5-fold more efficient to pellet a given particle (see “Experimental Procedures”), e.g.  $20,000 \times g$  supernatants in the TLA-110 are equivalent to  $100,000 \times g$  supernatants in SW32. Although normal PrP from uninfected cells remained in the supernatants (Fig. 5*A*, right panel), data in Fig. 5*A*, left panel, show that the vast majority ( $\sim 95\%$ ,  $n = 2$ ) of the PrP<sup>res</sup> in the soluble fraction is pelleted when *g* force was increased to  $100,000 \times g$  in the TLA-110 rotor (equivalent to  $500,000 \times g$  with SW32). We also performed ultracentrifugation of the soluble fraction through a 10% sucrose cushion. As expected for small aggregates, PrP<sup>res</sup> did not float in the sucrose and were found in the pellet (Fig. 5*B*). Cell-based assays showed that infectivity in the  $100,000 \times g$  supernatants (*Sup*<sup>100K</sup>) was 10-fold lower to that in *Sup*<sup>20K</sup> (data not shown). Collectively, these data indicate that infec-

## Small Infectious PrP<sup>res</sup> Aggregates

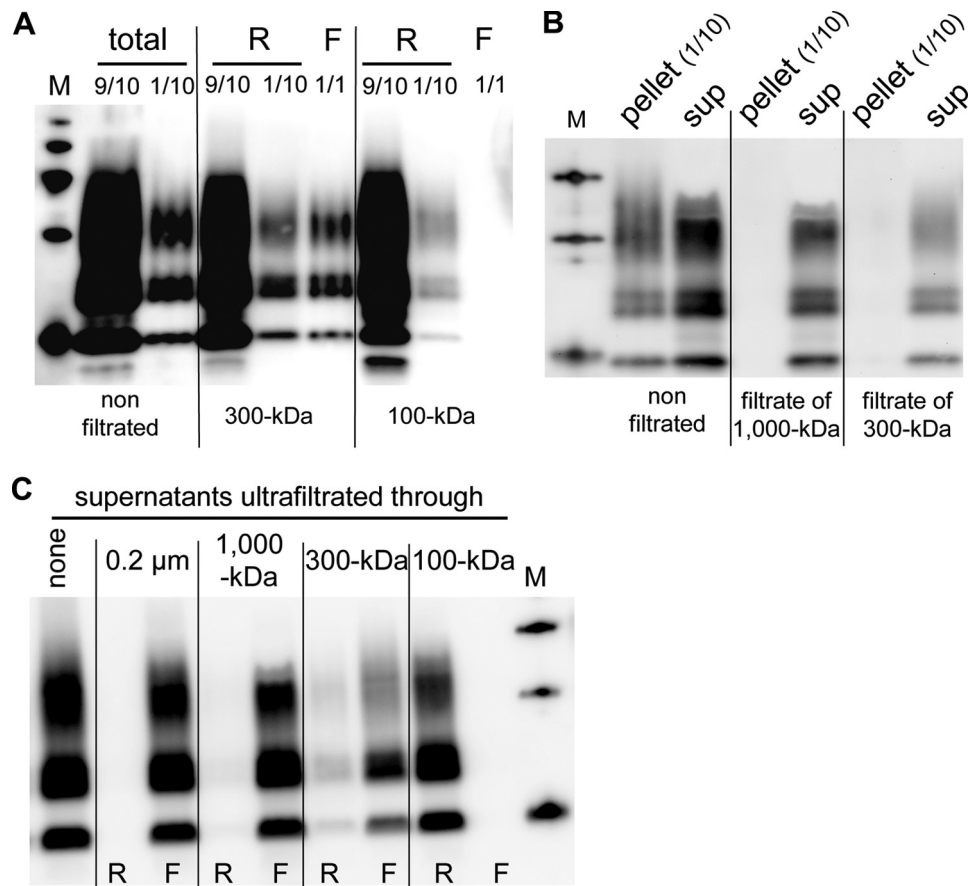


FIGURE 6. PrP<sup>res</sup> from soluble fractions passes through 300-kDa filters. *A*, analysis of PK-digested lysates before (*total*) or after ultrafiltration through 300-kDa or 100-kDa filters. Retentates (*R*; 1/10 and 9/10 were loaded) and the whole filtrates (*F*) were analyzed for PrP<sup>res</sup> by immunoblotting. *B*, filtrate fractions obtained after ultrafiltration through 1,000-kDa and 300-kDa filters of PK-digested lysates. Fractions were centrifuged to separate the insoluble (*pellet*) and soluble (*sup*) materials. Insoluble (1/10) and the whole soluble fractions from the filtrates were analyzed for PrP<sup>res</sup>. *C*, supernatants of centrifuged PK-digested lysates ultrafiltered through the indicated filters. PrP<sup>res</sup> in the retentates (*R*) and the filtrates (*F*) are shown.

tious PrP<sup>res</sup> species in the SW32 100,000 × *g* supernatants are not fully soluble.

We then subjected PK-digested cell lysates to ultrafiltration, a convenient alternative to sucrose gradient for separating large PrP<sup>res</sup> aggregates from other filterable molecules (24, 25). PrP<sup>res</sup> species in the retained (retentates) and nonretained (filtrates) fractions were analyzed by immunoblotting. Data in Fig. 6*A* show that although most of PrP<sup>res</sup> was retained, approximately 10% of total PrP<sup>res</sup> passed through 300-kDa but not through 100-kDa filters. Before ultrafiltration, insoluble PrP<sup>res</sup> is the major (~90%, Fig. 1) PK-resistant species. This was dramatically different in the filtrates from 1,000-kDa and 300-kDa membranes (Fig. 6*B*), indicating that PrP<sup>res</sup> in the soluble fraction passed much more readily through these filters than did insoluble PrP<sup>res</sup>. To confirm that the former quantitatively crosses filters of low molecular mass cutoff, PrP<sup>res</sup> from the soluble fraction was isolated by centrifugation and then submitted to ultrafiltration through a range of different filters. As expected, PrP<sup>res</sup> was not retained by 1,000- and 300-kDa filters as it was exclusively detected in the corresponding filtrates (Fig. 6*C*). In contrast, 100-kDa pore size fully retained PrP<sup>res</sup>. The above data indicate that the infectious PrP<sup>res</sup> recovered in the detergent-soluble fraction are small aggregates.

## DISCUSSION

After longstanding controversies, there is now a wide agreement that prion infectivity is associated with abnormally folded PrP. Historically, abnormal PrP has been operationally defined as PK-resistant and insoluble (26), and a wealth of studies indicates that fractions containing large insoluble PrP<sup>res</sup> aggregates are infectious. However, it does not imply that infectivity is exclusively associated with large aggregates of PrP<sup>res</sup> or that they are the more efficient species to promote infection. A number of studies reported successful disaggregation of at least part of prion infectivity by chemical and/or physical treatments. Early experiments showed that sonication of large PrP<sup>res</sup> aggregates (known as prion rods) in detergent and phospholipids can solubilize PrP<sup>res</sup> and infectivity (27). Denaturation by chaotropic salts and subsequent renaturation also led to infectivity recovery in low density fractions after sucrose gradients (28), and disruption of prion rods by sonication in detergents liberated infectivity in 100,000 × *g* supernatants (25). More recently, small PrP aggregates, isolated after physical disruption of detergent-extracted aggregates of PrP<sup>res</sup>, were shown to be more infectious than large ones (29), presumably by providing an increased number of multiplication-competent seeds. Although this study added strong support for small PrP aggre-



gates as more active species to initiate infection, firm evidence for the presence of small PrP<sup>res</sup> aggregates in infected tissues or cells was lacking. Thus, the biological significance of the aforementioned experimental treatments remained an open question. An important outcome of our study is to show that small infectious PrP<sup>res</sup> aggregates can be recovered from infected cells after a standard nondenaturing solubilization step in the absence of further treatments. Importantly, these small infectious PrP<sup>res</sup> aggregates were also observed in the absence of PK digestion, indicating that they were not derived from PK proteolysis of the large aggregates. They were conveniently separated from large PrP<sup>res</sup> aggregates by a solubility assay and/or by ultrafiltration through low molecular weight cutoff filters. Two lines of evidence indicate that the PrP<sup>res</sup> species in the detergent-soluble 100,000 × *g* supernatants are small PrP aggregates. First, and in contrast to normal PrP, these small PrP<sup>res</sup> species are eventually pelleted after ultracentrifugation at higher centrifugal forces. Second, they are immunoprecipitated by  $\beta$ S36, a mAb directed against aggregated PrP from infected tissue and not recognizing normal PrP (22). However, further purification will be required to determine their molecular composition.

The population of small PrP<sup>res</sup> aggregates, which represents roughly 10% of the total PrP<sup>res</sup> in infected Rov cells, is also observed in infected primary cultures of neurons and in brain tissue of infected mice, suggesting that it is produced by a wide variety of infected cells. An important issue is whether these small PrP<sup>res</sup> aggregates are more infectious than the large ones, as shown for small aggregates generated *in vitro* (29). Studies are now ongoing to address precisely this question. Another interesting point will be to determine whether the formation of small and large PrP<sup>res</sup> aggregates is due to different metabolic pathways. Alternatively, small aggregates could be metabolic intermediates in the formation of larger deposits. They could also be a cell-mediated catabolic product of large aggregates, contributing to an efficient transmission of infection during cell divisions (2). So far, and in contrast to yeast in which hsp104-dependent fragmentation is required for prion maintenance during successive cell divisions (30), no such activity has been identified for mammalian prions.

The discovery of small infectious PrP<sup>res</sup> aggregates further documents the diversity of abnormal PrP extracted from infected cells and tissues. Other small PrP aggregates have been detected and isolated in infected cells and tissues (11–15). In contrast to the small infectious PrP<sup>res</sup> aggregates described here, the previously described small PrP aggregates are sensitive to PK digestion, and no evidence indicates that they can transmit disease (31, 32). Small infectious PrP<sup>res</sup> aggregates, such as those described here, might give a molecular basis for the perplexing presence of infectivity in soluble preparations of infected hamster brain (33). Also, in a very recent study (10), density gradients were used to fractionate detergent-solubilized brain homogenate from *tg338* mice infected with the ovine prion agent (PG127) used here. Strikingly, fractions of low density, well separated from the bulk of large PrP<sup>res</sup> aggregates in heavier fractions, contained high levels of infectivity. The molecular basis of infectivity in the light fractions, not

addressed in Tixador's study, may involve the small PrP<sup>res</sup> aggregates characterized in our work.

Several neurodegenerative disorders, including Parkinson, Alzheimer, Huntington, and prion diseases, are characterized by the misfolding and the aggregation of specific proteins (34). Although early hypotheses considered the large insoluble aggregates as the only toxic entities, an important emerging view is that small soluble prefibrillar assemblies may be more efficient biological entities to promote cell death (35, 36). The toxic entities responsible for prion-mediated neuronal cell death and the underlying mechanisms are not yet clear (37, 38). The identification of naturally produced small PrP<sup>res</sup> aggregates in cultured neurons that will eventually undergo infection-dependent apoptosis (18, 39) may be useful to determine the potential neurotoxicity of these entities.

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