Normal and Scrapie-Associated Forms of Prion Protein Differ in Their Sensitivities to Phospholipase and Proteases in Intact Neuroblastoma Cells

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Previous studies have indicated that scrapie infection results in the accumulation of a proteinase K-resistant form of an endogenous brain protein generally referred to as prion protein (PrP). The molecular nature of the scrapie-associated modification of PrP accounting for proteinase K resistance is not known. As an approach to understanding the cellular events associated with the PrP modification in brain tissue, we sought to identify proteinase K-resistant PrP (PrP-res) in scrapie-infected neuroblastoma cells in vitro and to compare properties of PrP-res with those of its normal proteinase K-sensitive homolog, PrP-sen. PrP-res was detected by immunoblot in scrapie-infected but not uninfected neuroblastoma clones. Densitometry of immunoblots indicated that there was two- to threefold more PrP-res than PrP-sen in one infected clone. Metabolic labeling and membrane immunofluorescence experiments indicated that PrP-sen was located on the cell surface and could be removed from intact cells by phosphatidylinositol-specific phospholipase C and proteases. In contrast, PrP-res was not removed after reaction with these enzymes. Thus, either the scrapie-associated PrP-res was not on the cell surface or it was there in a form that is resistant to these hydrolytic enzymes. Attempts to detect intracellular PrP-res by immunofluorescent staining of fixed and permeabilized cells revealed that PrP was present in discrete perinuclear Golgi-like structures. However, the staining pattern was similar in both scrapie-infected and uninfected clones, and thus the intracellular staining may have represented only PrP-sen. Analysis of scrapie infectivity in cells treated with extracellular phospholipase, proteinase K, and trypsin indicated that, like PrP-res, the scrapie agent was not removed from the infected cells by any of these enzymes.

Prion protein (PrP) is a normal endogenous protein of unknown function found in cells of brain and a variety of other mammalian tissues (8, 11, 12, 17, 25, 26, 32). In the brains of humans and animals afflicted with Creutzfeldt-Jakob disease, kuru, scrapie, and other transmissible degenerative neuropathies, PrP accumulates in a proteinase K-resistant form (PrP-res) which can aggregate into fibrils (12, 13, 17, 23, 25, 32, 36) and be a component of amyloidlike plaques (19). {Several names have been used for forms of PrP derived from scrapie-infected tissues [PrP²⁷⁻³⁰ (5), PrP^{sc} (25), scrapie-associated fibril protein (13), Gp34 (33), and Sp 33-37 (3)] and uninfected tissues [PrP^c (25) and Cp 33-37 (3)]. Wishing to avoid terms which assume a relationship of the various forms of this protein to scrapie infectivity or specify a particular size for the protein, we have opted for the operational terms PrP-res and PrP-sen, which label two forms of the protein as they are distinguished experimentally by proteinase K digestion and can encompass the multiple molecular masses of the two forms.} This abnormal form of PrP has been identified as the major protein component of brain fractions containing the transmissible scrapie agent (4, 13, 23). Because the transmissible agents of scrapie and related diseases are highly resistant to treatments harmful to nucleic acids and because no agent-specific nucleic acids have been identified, it has long been postulated that these agents are devoid of nucleic acid and composed primarily of protein (1, 15, 27, 28). More recently, it was proposed that

PrP-res, or an aggregate thereof, is the transmissible agent of scrapie (4, 13, 24). However, it is not yet clear whether PrP-res is the transmissible scrapie agent itself, a component of the agent, or a by-product of either the infectious process or the disease.

The chemical basis for the difference between PrP-res and the proteinase K-sensitive PrP (PrP-sen) found in many normal cells and tissues is not known. Scrapie infection does not appear to modify the endogenous PrP gene of brain (2) or the size and quantity of PrP mRNA (8, 11, 26). Furthermore, expression of the PrP gene cloned from scrapie-infected mouse brain is not sufficient to generate PrP-res or the scrapie agent in mouse tissue culture cells (10). Thus, the PrP mRNA expressed in scrapie-infected brain does not appear to encode a unique scrapie-specific sequence. Rather, it seems more likely that posttranslational modification of PrP itself leads to the aggregation and accumulation of PrP-res in a proteinase K-resistant form. Several post-translational modifications of PrP are known to occur (6, 9, 10, 16, 22, 34), but none has been found to be scrapie specific. It is possible that proteinase K resistance does not require covalent modification but instead requires aggregation, conformational change, or association of PrP-sen with other components of scrapie-infected tissues.

To provide a system in which PrP and the scrapie agent can be studied in the absence of in vivo tissue pathology, we developed scrapie-infected mouse neuroblastoma clones with a high percentage of infected cells (29, 30). PrP can be labeled metabolically and immunoprecipitated from both uninfected (sc⁻) and scrapie-infected (sc⁺) neuroblastoma clones (29). The PrP detected in this manner in either type of

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clone is sensitive to proteinase K digestion in detergent lysates and, thus, by definition, is PrP-sen. Biosynthetic studies indicated that the mature PrP-sen of both the sc^- and sc^+ clones consists of two major glycosylated forms of 30 and 35 to 41 kilodaltons (kDa) which are anchored at the cell surface by covalent linkage to phosphatidylinositol (9). No scrapie-associated differences in PrP-sen biosynthesis were observed. Recently, however, the detection of PrP-res in scrapie-infected mouse neuroblastoma cells by immunoblotting was reported (7). Here we report the detection of PrP-res in similar sc^+ neuroblastoma cells and show, in addition, that PrP-res in intact cells differs from PrP-sen in its sensitivity to proteases and phosphatidylinositol-specific phospholipase C (PIPLC).

MATERIALS AND METHODS

Neuroblastoma cells. The neuroblastoma cell clones 321, 322, and 324 used in these experiments are subclones of the 29-161 clone described in detail before (29, 30). The scrapie infectivity in the 322 clone is equivalent to that of the parent 29-161 culture, and recipient mice die in ~180 days after inoculation of 10^5 cells of either clone. Scrapie infectivity has never been detected in clones 321 and 324.

Antibodies. A synthetic peptide fragment of the PrP amino acid sequence (peptide 1b) (36) was provided by Michael Buchmeier of Scripps Clinic and Research Foundation, La Jolla, Calif. This peptide was conjugated to keyhole limpet hemocyanin and inoculated into rabbits by a protocol described previously (36). A portion of the whole anti-PrP peptide rabbit serum (no. 9) obtained was affinity purified, using a protocol provided by Michael Buchmeier (26a) as follows. A 10-mg portion of peptide 1b in 10 ml of 0.5 M NaCl–0.1 M Tris hydrochloride, pH 7.5, at 20°C (buffer A) was bound to 0.5 g of thiopropyl-Sepharose (Pharmacia, Uppsala, Sweden) swollen in buffer A by agitating for 1 h at room temperature. The Sepharose beads were pelleted and washed twice with buffer A. The beads were mixed with 10 ml of 0.1 M sodium citrate, pH 4.5, and 5 μ l of 2-mercaptoethanol for 1 h at room temperature, washed extensively with buffer A on a sintered-glass filter, suspended in phosphate-buffered saline (PBS), and packed in a column. Clarified anti-PrP peptide serum (2 ml) was passed through the column, and the column was washed with PBS until the A_{280} of the effluent was stable. Bound antibodies were eluted with 6 ml of 0.1 M sodium citrate, pH 3.0, immediately neutralized to pH 7 to 8 with 1 M Tris base, dialyzed against PBS, and then concentrated to the original serum volume. A polyclonal rabbit antiserum (R073) raised against hamster PrP 27-30 was provided by S. Prusiner, University of California, San Francisco.

Detection of PrP-res by immunoblotting. We were previously unable to detect PrP-res in our cloned sc^+ neuroblastoma cells (29). However, after modifications of the cell extraction, proteinase K treatment, and protein precipitation procedures, we were able to detect PrP-res in this clone of sc^+ neuroblastoma cells. PrP-res was detected in extracts of sc^+ neuroblastoma cells as follows. Confluent monolayers of cells in 75-cm² Corning tissue culture flasks (~10⁷ cells), or cells in suspension after treatments described above, were washed once with phosphate-buffered balanced salts solution (PBBS) and lysed in 3 ml of 0.5% Nonidet P-40–0.5% sodium deoxycholate–150 mM NaCl–10 mM EDTA–50 mM Tris hydrochloride, pH 7.4, per 75-cm² flask equivalent. This lysate was centrifuged at $1,000 \times g$ for 10 min. The supernatant fluid was removed and treated with 100 μ g of protein-

ase K per ml for 1 h at 37°C with occasional agitation. The digestion was terminated by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM and cooling on ice for 30 min. Residual proteins were extracted and precipitated with methanol-chloroform by a previously described method (35). The protein pellet was air dried and suspended in 40 to 50 μ l of sample buffer (20) containing 5% sodium dodecyl sulfate (SDS). The suspension was sonicated for 60 s at maximum power in a cup horn apparatus (Heat Systems-Ultrasonics, Farmingdale, N.Y.), boiled for 2 min, and cleared by microcentrifugation for 2 min at $11,000 \times g$ prior to SDS-polyacrylamide gel electrophoresis (PAGE). The SDS-PAGE and transfer of proteins onto nitrocellulose filters have been described before (29). More recently, we have substituted Immobilon P (Millipore Corp., Bedford, Mass.) for nitrocellulose because we have found it to be several times more efficient at binding PrP and other proteins. The primary antibody (whole rabbit anti-PrP peptide serum, 1:2,000 dilution, unless indicated otherwise) was incubated with the nitrocellulose or Immobilon filter for 2 h at room temperature, and then the filters were developed with a standard kit, using alkaline phosphatase-conjugated goat anti-rabbit antiserum (Promega ProtoBlot W 3930).

Detection of PrP-sen by immunoblot. Media from the treatment of intact cells with PIPLC as described below were cooled on ice and centrifuged at $1,000 \times g$ for 5 min at 4°C to pellet dislodged cells and debris. The supernatant was treated with PMSF (0.5 mM), pepstatin (0.7 μ g/ml), leupeptin (0.5 μ g/ml), and EDTA (5 mM). Bovine serum albumin (20 μ g/ml) was added as a carrier, and proteins were extracted with methanol-chloroform and prepared for immunoblot analysis as described above.

PIPLC and protease treatments of intact cells. Confluent clone 322 cells were rinsed once and dislodged quickly in 2 mM EDTA–5 mM glucose in PBS by striking the flask laterally on a hard object. The cells were then transferred into tubes containing 0.2 volume of fetal bovine serum (FBS) and 1 volume of ice-cold PBBS. The cells were washed twice in PBBS and, unless stated otherwise, were suspended in 3 ml, per flask equivalent, of (i) PBBS containing PIPLC from *Bacillus thuringiensis* (21; the generous gift of Martin Low, Columbia University College of Physicians and Surgeons, New York, N.Y.) at an activity of 1.6 μ mol/min per ml with [³H]phosphatidylinositol as substrate, (ii) PBBS containing 100 μ g of proteinase K per ml, or (iii) 0.5 mg of trypsin (1:250; Difco Laboratories, Detroit, Mich.) per ml in 136 mM NaCl–5 mM KCl–5 mM glucose–7 mM NaHCO₃–0.6 mM EDTA. The cells were incubated for 40 (37°C), 10 (ambient temperature), and 10 or 20 (ambient temperature) min for the PIPLC, proteinase K, and trypsin treatments, respectively. The reactions were stopped by chilling the cells on ice and adding 0.2 volume of FBS containing 5 mM PMSF unless the cells were to be assayed for PrP-res by immunoblotting, in which case the PMSF was omitted from the FBS and subsequent washes. The cells were then washed three times with PBBS containing 2% FBS–0.5 mM PMSF before subsequent analyses.

Release of metabolically labeled PrP from intact cells by PIPLC, trypsin, and proteinase K. Neuroblastoma cells were metabolically labeled in 25-cm² plastic flasks (Corning) with L-[³⁵S]methionine (Dupont, NEN Research Products, Boston, Mass.) for 30 min and chased with medium containing methionine for 150 min as described previously (9). The intact cells were then incubated with PIPLC, trypsin, or proteinase K as described above, washed twice in minimal essential medium, and lysed in lysing buffer (29) supple-

mented with 1 mg of soybean trypsin inhibitor per ml and 1 mM PMSF. PrP was detected in the lysates and in the PIPLC medium by immunoprecipitation, SDS-PAGE, and fluorography as described previously (29). For quantitation of the labeled PrP by laser densitometry, the film was preflashed to increase the linearity of the film response to fluorographic signals (Review 23: Radioisotope Detection by Fluorography and Intensifying Screens, Amersham Corp., Arlington Heights, Ill.).

Membrane immunofluorescence detection of PrP. Neuroblastoma cells, in suspension after the PIPLC or protease treatments described above, were pelleted at $1,000 \times g$ for 5 min and washed twice in membrane immunofluorescence buffer (MIB; PBBS with 2% FBS, 0.5 mM PMSF, and 0.01 M sodium azide) and filtered through Nitex polyamide nylon fiber mesh 3-112/40xx (Tekto, Inc.). The washed and filtered cells (10^5) were suspended in 40 μ l of MIB, combined with 40 μ l of MIB containing 1:40-diluted affinity-purified anti-PrP peptide, and placed on ice for 30 min in 96-well microdilution plates. After rinsing twice with MIB, the cells were incubated for 30 min on ice in MIB containing 1:15-diluted fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika, Malvern, Pa.). The cells were washed twice in MIB and suspended in 50% glycerol-2% Formalin in PBS. Membrane fluorescence was monitored on a Leitz fluorescence microscope and a flow cytometer. For flow cytometry, 10,000 events were analyzed by a Becton Dickinson FACStar equipped with an argon ion laser emitting 488-nm light at 200 mW. The instrument was operated in log gain on the fluorescence channel, with full-scale fluorescence being 4 log decades. Forward-angle light scatter gates were set to exclude acellular debris.

Cytoplasmic immunofluorescence detection of PrP. Neuroblastoma cells were seeded in Linbro six-well tissue culture plates at 10^5 cells per well and used 1 or 2 days afterwards. The cells were fixed in PBBS containing 1.5% formaldehyde (freshly diluted from a 37% formaldehyde stock) for 30 min, rinsed twice with PBBS, and incubated in PBBS containing 100 mM glycine-0.1% bovine serum albumin for 30 min. The cells were rinsed twice in PBBS, permeabilized with a 3-min incubation in PBBS containing 0.4% (vol/vol) Triton X-100, and again rinsed twice in PBBS. The cells were then incubated for 30 to 60 min in 200 μ l, per well, of a 1:100 dilution of affinity-purified anti-PrP peptide 1b in PBBS supplemented with 2% FBS and 10 mM sodium azide. After rinsing three times with the antibody diluent, the cells were incubated under the same conditions with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. After three more rinses in diluent, the cells were mounted in 50% glycerol-2% Formalin and observed under a fluorescence microscope.

Assay of scrapie infectivity. Flasks (75 cm²) of sc⁺ clone 29-161 cells were treated with PIPLC or trypsin as described above. Thereafter, the cells were counted, frozen, thawed, and sonicated. Cellular debris was removed by centrifuging at $1,000 \times g$ for 5 min. The media from the PIPLC treatments were collected, and detached cells were removed by centrifuging at $1,000 \times g$ for 5 min. The medium samples were then centrifuged at $140,000 \times g$ for 60 (experiment 1) or 180 (experiment 2) min. The supernatants were removed and the pellets were suspended in 1 ml of PBS by sonicating and vortexing. Scrapie infectivity titers in the cleared cell lysates, medium pellets, and medium supernatants were determined by endpoint dilution bioassay in mice as described previously (29). The 50% lethal doses and standard errors

FIG. 1. Detection of PrP-res in sc⁺ neuroblastoma cells. sc⁻ (clone 321) and sc⁺ (clone 322) neuroblastoma clones were lysed with detergent, treated with proteinase K, and immunoblotted, using anti-PrP peptide 1b, anti-PrP peptide 1b preabsorbed with synthetic peptide 1b, or anti-PrP 27-30. Some 1.5×10^7 cell equivalents were loaded into each lane. Bands containing the specific PrP peptide epitope were those which disappeared when the anti-PrP peptide was preabsorbed. The major band stained by the antisera in both the sc⁻ and sc⁺ clones which was not removed by preabsorption of the antiserum with peptide 1b appeared to be proteinase K since immunoblotting of proteinase K alone produced a similar band (data not shown). The estimated molecular masses (in kilodaltons) of the specific PrP bands in the neuroblastoma cell extracts are indicated on the right.

were estimated by using the method of Spearman and Karber as modified by Irwin and Cheeseman (14).

RESULTS

Detection of PrP-res. To study the relationship between PrP-res and scrapie infectivity, we sought to determine whether PrP-res could be detected *in vitro*, i.e., in scrapie-infected tissue culture cells, as it is in scrapie-infected tissues. Detergent extracts of sc⁻ and sc⁺ mouse neuroblastoma cell clones were treated with proteinase K, and, resistant proteins were visualized by immunoblot, using rabbit antiserum against peptide 1b. Proteinase K-resistant bands unique to the sc⁺ clone were observed (Fig. 1). Preabsorption of the primary antibody with the synthetic PrP peptide antigen abolished the staining of two major bands with apparent molecular masses of 19 and 23 kDa and a more diffuse group of bands centered around 28 kDa, indicating that these bands contained the PrP peptide epitope. These apparent PrP bands comigrated with the proteinase K-treated PrP-res bands from scrapie-infected mouse brain (not shown), and the apparent molecular mass values were similar to those reported previously for PrP-res from scrapie mouse brain (18) and sc⁺ neuroblastoma cells (7). The same bands were also stained with an antiserum raised against 27- to 30-kDa PrP-res from scrapie-infected hamster brain (Fig. 1) but not with normal rabbit serum (not shown). Thus, based on the immunoreactivity, the apparent molecular masses, and the proteinase K resistance of the 19-, 23-, and 28-kDa bands, we concluded that they were PrP-res.

Resistance of PrP-res in intact sc⁺ cells to PIPLC and proteases. Having detected scrapie-associated PrP-res in tissue culture cells, we sought ways to discriminate PrP-res from PrP-sen which might suggest differences in posttranslational modification or subcellular localization or both.

FIG. 2. Resistance of PrP-res in intact cells to digestion by PIPLC, proteinase K, and trypsin. Lysates of control sc^+ neuroblastoma cells (clone 322) or cells treated with PIPLC, proteinase K (PK), or trypsin were digested with proteinase K and extracted. Extracts were subjected to SDS-PAGE and immunoblotted onto Immobilon as described in Materials and Methods. Each sample contained 3×10^6 cell equivalents. In addition, to show the sensitivity of the immunoblot assay to the amount of PrP-res in the sample, dilutions of the trypsin-treated sample (designated as the fraction of the undiluted sample) were included. The positions of the specific PrP bands are designated on the left in kilodaltons. The dark spot encroaching on the left side of the untreated lane at ~ 28 kDa was spillover from a molecular mass marker in the adjacent lane.

Since we have shown qualitatively in a previous study that metabolically labeled and immunoprecipitable PrP-sen could be digested on the surface of intact cells by PIPLC and protease treatments (9), we tested whether the same was true for PrP-res. Intact sc^+ cells were treated with PIPLC, proteinase K, or trypsin before lysis and were analyzed for PrP-res by immunoblotting (Fig. 2). The amount of PrP-res detected in the cells was not affected by any of these treatments. Dilutions of cell lysate indicated that even a twofold fold reduction of PrP-res in the cells would have been detected readily by this procedure (Fig. 2). Additional experiments showed that PrP-res in intact cells was similarly resistant to two other broadly acting proteases, pronase and papain (data not shown). This resistance of PrP-res to PIPLC and the proteases in intact cells indicated that either PrP-res was not on the cell surface or that it existed there in a state that was resistant to these hydrolytic enzymes.

Sensitivity of PrP-sen to phospholipase and proteases. To compare the protease and PIPLC sensitivities of PrP-sen and PrP-res in intact sc^+ neuroblastoma cells, it was necessary to quantitate the proportion of PrP-sen that was susceptible to these enzymes. Thus, we expanded on our previous qualitative studies of the release of metabolically labeled PrP by PIPLC and trypsin (9) by adding a proteinase K treatment and by quantitating the proportion digested by each treatment, using densitometry. sc^+ cells were pulse-labeled with [35 S]methionine and chased for a period which, based on our earlier study (9), was sufficient to allow translocation of labeled PrP-sen to the cell surface. Following treatments of the intact cells with PIPLC, trypsin, or proteinase K, the proportion of the original 35 S-labeled PrP-sen remaining in the cells was estimated by immunoprecipitation, fluorog-

FIG. 3. Release of metabolically labeled PrP-sen from intact sc^+ neuroblastoma cells by PIPLC, proteinase K (PK), and trypsin (T). Clone 322 cells were pulse-chase labeled with [35 S]methionine, dislodged, and treated with PIPLC, proteinase K, or trypsin as described in Materials and Methods. Control cells (C) were treated identically to the PIPLC-treated cells except that the PIPLC was omitted from the treatment buffer. The relative amounts of labeled PrP immunoprecipitated from lysates of treated and control neuroblastoma cells were estimated by laser densitometry of preflashed fluorography film. Densitometric traces spanning the 30- and 35- to 41-kDa PrP bands (9, 29) were obtained by using an LKB Ultrascan laser densitometer. The areas under the peaks were quantitated by cutting out the peaks and weighing them. The low-level contributions of non-PrP proteins to the peaks was eliminated by subtracting the signal obtained from lanes of analogous samples precipitated by the antiserum preabsorbed with the peptide antigen. Bars represent means $\pm$ standard errors of values from two independent experiments.

raphy, and laser densitometry of fluorography films. Figure 3 shows that $\sim 90\%$ of both the labeled 30- and 35- to 41-kDa PrP bands were removed from sc^+ cells with these enzymes. Thus, most of the immunoprecipitable, [35 S]methionine-labeled PrP was chased to the cell surface in a state that was sensitive to proteases and PIPLC.

To confirm these results and to determine whether the metabolically labeled PrP was representative of most of the immunoreactive PrP on the cell surface, we performed membrane immunofluorescence studies. Affinity-purified anti-PrP peptide antibodies readily detected PrP on the surface of live neuroblastoma cells (Fig. 4). Preabsorption of the primary antibody with peptide 1b reduced the fluorescence of the cells to near-background levels, indicating that most of the staining was specific for the PrP peptide epitope. To determine whether the immunoreactive PrP on the cell surface was sensitive to proteinase K, trypsin, and PIPLC, we pretreated the cells with these enzymes prior to immunofluorescent labeling. A representative histogram of proteinase K-pretreated cells is included in Fig. 4 to show that it was nearly superimposed on the background peak. To obtain estimates of the proportions of the immunoreactive PrP removed by proteinase K, trypsin, and PIPLC treatments, specific mean fluorescence intensities of the membrane fluorescence-labeled cell populations were compared (Fig. 5). Trypsin and proteinase K pretreatments reduced the

FIG. 4. Flow cytometric analysis of cell surface PrP in sc^+ neuroblastoma cells. Clone 322 cells were treated with PBBS alone (•••) or PBBS containing 50 μ g of proteinase K per ml (—), then incubated with affinity-purified anti-PrP peptide and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin, and analyzed by flow cytometry as described in Materials and Methods. Also included are PBBS-treated cell controls in which the primary antibody was omitted (••••) or preabsorbed with PrP peptide 1b (—•—).

specific mean fluorescence intensity of both sc^- and sc^+ clones by at least 95%, while the PIPLC treatments reduced the specific mean fluorescence intensity by approximately 75%. The residual specific fluorescence of the cells after

FIG. 5. Release of membrane immunofluorescence-labeled PrP from intact sc^- and sc^+ neuroblastoma cells by PIPLC, proteinase K (PK), and trypsin (T). Clones 321 (sc^-) and 322 (sc^+) were dislodged and treated with the PIPLC (0.8 and 1.6 μ mol/min per ml), proteinase K (50 and 100 μ g/ml), trypsin (500 μ g/ml), or PBBS alone (C). The cells were labeled with affinity-purified anti-PrP peptide and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G and analyzed with a flow cytometer. Specific mean fluorescence intensities were calculated as the difference between the total mean fluorescence intensity of the cells stained with anti-PrP peptide and the mean fluorescence intensity of cells from the same treatment stained with the antibody preabsorbed with peptide 1b. Because the fluorescence units for the positive control varied between experiments, the results from independent experiments were normalized and presented as percent specific mean fluorescence of the control cells. Values are means $\pm$ standard errors of values obtained from at least two independent experiments.

FIG. 6. Relative amounts of PrP-res and PrP-sen in sc^+ neuroblastoma clone 322. Confluent cells in a 150-cm² flask were treated with PIPLC, dislodged in PBS-EDTA, lysed with detergent, and treated with proteinase K. Proteins in the proteinase K-treated lysate and the PIPLC treatment medium were extracted and immunoblotted, using Immobilon and either affinity-purified anti-PrP peptide 1b antibody or the same antibody preabsorbed with synthetic peptide 1b. Approximately 3×10^6 (lane 1) and 10^6 (lane 2) cell equivalents of PIPLC medium extract and 10^6 cell equivalents of lysate (lane 3) were loaded onto the gel. The positions of the PrP-sen bands (30 and 35 kDa) and PrP-res bands (19, 23, and 28 kDa) are designated on the left.

PIPLC treatment was not affected by increasing the activity of the enzyme and, as noted above, may represent an inherently PIPLC-resistant PrP subfraction or an artifact of the treatment procedure. These immunofluorescence data provided evidence to confirm the immunoprecipitation experiments showing that most of the PrP-sen is sensitive to the proteinase K, trypsin, and PIPLC on the surface of intact cells. This is in contrast to PrP-res, whose detection by immunoblotting was unaffected by these treatments. The observation that similar percentages of the PrP remained on the cell surface after treatments of both sc^- and sc^+ cells suggested that the residual fluorescence in sc^+ cells was not due primarily to PrP-res.

Relative amounts of PrP-res and PrP-sen in sc^+ cells. To test whether a portion of the residual cell surface immunofluorescence observed after protease and PIPLC treatments of intact sc^+ neuroblastoma cells represented PrP-res, we estimated the relative proportions of PrP-res and PrP-sen in the cells. The inability to detect PrP-sen cleanly in crude cell lysates by immunoblot prevented a direct comparison of PrP-sen and PrP-res in lysates. However, PrP-sen was detected by immunoblot in the medium of PIPLC-treated sc^+ cells (Fig. 6). The apparent molecular masses of the observed PrP bands at 30 and 35 kDa were the same as those of PrP-sen observed by metabolic labeling and immunoprecipitation (9, 29). Preabsorption of the antibody with peptide 1b eliminated the staining of these bands preferentially, indicating that they contained the PrP peptide epitope. Other experiments showed that treatment of the medium with as little as 5 μ g of proteinase K per ml for 15 min at ambient temperature eliminated these PrP bands, confirming that they were PrP-sen (data not shown). Immunofluorescent labeling and flow cytometric analysis of samples of the cells used in Fig. 6 indicated that PIPLC and proteinase K treatments of the intact cells reduced the cell surface PrP

fluorescence signal by 72 and 98%, respectively. Thus, it was assumed that the PrP detected in the PIPLC medium represented ~72% of the total immunoreactive PrP on the cell surface. As was the case in the experiment shown in Fig. 2, the PIPLC treatment of intact cells did not affect the level of PrP-res detected in the cells by immunoblot (not shown). Therefore, the relative amounts of PrP-res and PrP-sen in these cells was estimated by comparing the immunoblot staining of PrP-sen in the medium of PIPLC-treated cells with PrP-res in the cell lysates (Fig. 6). Quantitative analysis of the staining by laser densitometry indicated that there was two- to threefold more PrP-res than PrP-sen in these cells. Thus, the residual PrP fluorescence left on the cell surface after PIPLC (28%) and protease (2%) treatments was too low to account for a major proportion of the PrP-res detected by immunoblot. This suggests that most or all of the PrP-res was intracellular or was not efficiently recognized on the cell surface by the anti-PrP peptide antibodies.

Detection of cytoplasmic PrP. Given the above evidence that PrP-res may be primarily intracellular, we used indirect immunofluorescence with anti-PrP antibodies to localize PrP in fixed and permeabilized sc^+ and sc^- neuroblastoma cells (Fig. 7). In both cell types, the fixation and permeabilization procedures eliminated the cell surface signal; however, discrete cytoplasmic structures stained with the affinity-purified anti-PrP peptide antibodies (Fig. 7A to C). This staining was not observed when the antibody was preabsorbed with the synthetic PrP peptide antigen (Fig. 7D to F). The stained structures had the morphological appearance of Golgi bodies in that they had a polar perinuclear distribution. Since PrP is a plasma membrane glycoprotein and therefore undergoes carbohydrate processing in the Golgi body during its biosynthesis (9), the presence of PrP staining in the Golgi body would not be surprising.

No difference was observed in the staining of most of the cells of the sc^- and sc^+ cultures (Fig. 7A and B). However, the staining pattern appeared more dispersed in some cells of the sc^+ cultures (Fig. 7C). The more dispersed appearance was usually associated with cells of quite broad and flattened morphology. Immunoblot analysis of the sc^+ cells used in the experiment in Fig. 7 indicated that they contained one- to three-fold more PrP-res than PrP-sen (data not shown). This suggests that, if PrP-res occupied a unique intracellular location and were recognized in its native state by the anti-PrP peptide antibody, we would have detected it. However, since similar structures were stained in both sc^- and sc^+ cultures, it was not possible to determine whether the staining observed in the sc^+ cells represented PrP-sen alone or a combination of PrP-sen and PrP-res.

Resistance of scrapie infectivity to PIPLC and trypsin in intact sc^+ cells. The above experiments demonstrated that two forms of PrP could be separated by treating intact sc^+ neuroblastoma cells with proteases or PIPLC. Since PrP has been postulated to be a component of scrapie infectivity, it was of interest to determine whether, with respect to protease and PIPLC sensitivity, scrapie infectivity correlated with PrP-sen or PrP-res in these sc^+ tissue culture cells. Live sc^+ neuroblastoma cells were treated with PIPLC or trypsin, washed, homogenized, and then injected into susceptible

TABLE 1. Effect of PIPLC and trypsin on scrapie infectivity in intact sc^+ neuroblastoma cells

Treatment	Scrapie infectivity ($\log_{10}$ LD ₅₀ $\pm$ SEM) ^a	
	Expt 1	Expt 2
Control	3.60 $\pm$ 0.19	2.80 $\pm$ 0.18
PIPLC	3.80 $\pm$ 0.25	2.30 $\pm$ 0.33
Trypsin	3.40 $\pm$ 0.19	2.50 $\pm$ 0.19

^a Infectivity in lysates of 4×10^5 cells (LD₅₀, 50% lethal dose). Values determined from two independent experiments are shown. The media from the treatments were also collected, ultracentrifuged to concentrate any infectivity released from the cells, and assayed for infectivity. However, no infectivity (<1 LD₅₀ in medium fractions derived from treatment of 4×10^5 cells) was detected in either the medium pellets or the supernatants.

mice. Neither treatment significantly reduced the scrapie titers in the cells (Table 1), nor was infectivity detected in the media after the treatments. Thus, like PrP-res, the scrapie infectivity was not affected by treatments which removed immunoreactive PrP-sen from intact neuroblastoma cells.

DISCUSSION

If the generation of PrP-res is directly related to scrapie agent replication, it should be observed in tissue culture cells replicating the scrapie agent as well as in scrapie-infected animals. The results of this study provide evidence in support of an earlier report (7) that PrP-res is present in sc^+ but not sc^- neuroblastoma cells in vitro and thus correlates with the presence of the scrapie agent. However, while this finding suggests that scrapie tissue pathology in animals is not necessary for PrP-res generation, it does not eliminate the possibility that the formation of PrP-res is secondary to the presence or replication of the scrapie agent. For instance, a scrapie-encoded or scrapie-modified protein(s) could influence the biosynthesis and metabolism of endogenous PrP without the resulting modified PrP (PrP-res) being a component of the infectious agent. Further studies will be necessary to address that possibility.

Since the presence of PrP-res correlates with scrapie infectivity in neuroblastoma cells and in other systems, it is important to understand the difference between PrP-sen and PrP-res as well as the possible role of PrP-res in scrapie agent replication or pathology or both. The ability to detect both PrP-sen and PrP-res in sc^+ neuroblastoma clones has enabled us to begin studying these topics in vitro. In the present study, we have demonstrated that PrP-sen and PrP-res differ in their sensitivities to PIPLC and proteases in intact cells, the latter being insensitive to these enzymes. There are several possible explanations for these differences. For example, whereas mature metabolically labeled PrP-sen is found on the cell surface (9, 10), PrP-res may be intracellular and thus unavailable to extracellular enzymes. Alternatively, PrP-res may be on the cell surface, but in a state in which the phospholipase and protease cleavage sites are protected. This could be due to an altered conformation, posttranslational modification, or aggregation of PrP-res. The precise localization of PrP-res in the sc^+ neuroblastoma

FIG. 7. Cytoplasmic immunofluorescent labeling of PrP in neuroblastoma cells. sc^- (clone 324; panels A and D) and sc^+ (clone 322; panels B, C, E, and F) neuroblastoma cultures were fixed with formaldehyde, permeabilized with Triton X-100, and stained by using either affinity-purified anti-PrP peptide antibody (panels A to C) or the same antibody preabsorbed with peptide 1b (panels D to F) as the primary antibody. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was the secondary antibody in all cases. The fluorescence staining was photographed at $\times 1,280$ magnification on a fluorescence microscope.

clones has been hampered by the lack of antibodies known to react with this species in its native state. Although the anti-PrP peptide antiserum clearly recognizes PrP-res on immunoblot filters after denaturation in SDS-PAGE, we have no evidence that it binds to PrP-res under the milder conditions used for immunofluorescence or immunoprecipitation. Thus, further localization studies may require the use of other antibodies specific for PrP-res or the development of cell treatments which do not destroy cellular morphology but sufficiently denature PrP-res to make it reactive with our present antibodies.

The reason for the presence of a subpopulation of cells in the sc^+ cultures which have a broad and flattened shape and a dispersed cytoplasmic PrP staining pattern is unknown. While it is possible that these cells represent high scrapie producers within the cultures, they may be merely morphological variants which are unrelated to scrapie infection.

It is interesting that, in both sc^- and sc^+ neuroblastoma clones, about 25% of the fluorescence-labeled PrP-sen on the surface of neuroblastoma cells was resistant to PIPLC digestion. This suggests that a portion of the normal cell surface PrP may lack the glycosyl phosphatidylinositol anchor or may have a modified anchor as has been observed with acetylcholine esterase (31). Alternatively, aggregation of the cells after EDTA treatment may have protected some of the cell surface PrP from PIPLC digestion because, unlike trypsin and proteinase K, PIPLC may be unable to disaggregate cells on its own.

The finding that scrapie infectivity remained associated with the neuroblastoma cells during treatments which remove mature PrP-sen from the cell is consistent with the idea that two different forms of PrP exist in these cells and that only one of them, PrP-res, appears to correlate with scrapie infectivity. However, this correlation does not prove a structural association between PrP-res and the infectious agent.

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