

# Scrapie Prion Proteins Accumulate in the Cytoplasm of Persistently Infected Cultured Cells

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**Abstract.** The cellular prion protein (PrP<sup>C</sup>) is a sialoglycoprotein anchored to the external surface of cells by a glycosyl phosphatidylinositol moiety. During scrapie, an abnormal PrP isoform designated PrP<sup>Sc</sup> accumulates, and much evidence argues that it is a major and necessary component of the infectious prion. Based on the resistance of native PrP<sup>Sc</sup> to proteolysis and to digestion with phosphatidylinositol-specific phospholipase C as well as the enhancement of PrP<sup>Sc</sup> immunoreactivity after denaturation, we devised *in situ* immunoassays for the detection of PrP<sup>Sc</sup> in cultured cells. Using these immunoassays, we identified the sites of PrP<sup>Sc</sup> accumulation in scrapie-infected cultured cells. We also used these immunoassays to isolate PrP<sup>Sc</sup>-producing clones from a new hamster brain cell line (HaB) and found an excellent correlation be-

tween their PrP<sup>Sc</sup> content and prion infectivity titers. In scrapie-infected HaB cells as well as in scrapie-infected mouse neuroblastoma cells, most PrP<sup>Sc</sup> was found to be intracellular and most localized with ligands of the Golgi marker wheat germ agglutinin. In one scrapie-infected HaB clone, PrP<sup>Sc</sup> also localized extensively with MG-160, a protein resident of the medial-Golgi stack whereas this colocalization was not observed in another subclone of these cells. Whether the sites of intracellular accumulation of PrP<sup>Sc</sup> are limited to a few subcellular organelles or they are highly variable remains to be determined. If the intracellular accumulation of PrP<sup>Sc</sup> is found in the cells of the central nervous system, then it might be responsible for the neuronal dysfunction and degeneration which are cardinal features of prion diseases.

**D**ETECTION and quantification of scrapie infectivity currently relies on bioassays performed in laboratory rodents (Chandler, 1961; Prusiner et al., 1980, 1982). Although bioassays using incubation time measurements are more rapid and economical than end-point titrations, they still require several months or even years depending on the prion isolate (Gajdusek, 1977).

In contrast to prion research, where the infectious particles are replicated and assayed in animals, most viruses can be readily propagated and titrated in cultured cells (Dulbecco, 1952). Numerous advances in virology can be traced to the use of cultured cells (Enders et al., 1949).

During the past two decades, various tissue culture systems have been described in which scrapie or Creutzfeldt-Jakob disease (CJD)<sup>1</sup> prions replicate at low levels (Clarke and Haig, 1970; Clarke and Millson, 1976; Markovits et al., 1981, 1982; Rubenstein et al., 1984; Race et al., 1987; Butler et al., 1988). As no well-defined scrapie-specific effects (cytopathic or others) have been consistently observed in these cultures, *in situ* assays analogous to the plaque or focus

assays used in virology have not been developed. Thus, the study of prion replication and transmission in tissue culture has remained dependent on animal bioassays which, apart of being extremely slow, cannot render the detailed information obtained by plaque assays for viruses. The lack of rapid *in situ* methods for the detection of scrapie-infected cells has therefore hampered the development of cell culture systems for replication of prions and the study of their properties.

Considerable evidence from biochemical, immunologic, pathologic, and genetic studies argues persuasively that PrP<sup>Sc</sup> is a major and necessary component of the infectious prion particle (Prusiner, 1987, 1989; Scott et al., 1989; Westaway et al., 1989). On this basis, we thought that PrP<sup>Sc</sup> was the best candidate to serve as a chemically identifiable marker for scrapie in cells. The persistent infection of mouse neuroblastoma (N<sub>2</sub>a) cells with mouse scrapie and CJD, and the subsequent isolation of infectious subclones, has been recently achieved (Butler et al., 1988; Race et al., 1988). In our laboratory, a perfect correlation was observed between the prion infectivity of the subclones and their PrP<sup>Sc</sup> content (Butler et al., 1988). Detection of PrP<sup>Sc</sup> was performed using Western immunoblots after limited proteinase K digestion. This procedure requires preparation and digestion of an extract prepared from ~10<sup>6</sup> cells, making the screening of large numbers of clones for PrP<sup>Sc</sup> extremely cumbersome, and illustrating the need for a sensitive *in situ* method for PrP<sup>Sc</sup> detection.

1. *Abbreviations used in this paper:* CJD, Creutzfeldt-Jakob disease; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; PIPLC, phosphatidylinositol-specific phospholipase C; PrP, prion protein; PrP<sup>C</sup>, cellular isoform of the prion protein; PrP<sup>Sc</sup>, scrapie isoform of the prion protein; TBST, 0.05% Tween 20, 100 mM NaCl, 10 mM Tris, pH 7.8; WGA, wheat germ agglutinin.

PrP<sup>Sc</sup> is known to be an abnormal isoform of a normal membrane glycoprotein designated PrP<sup>C</sup> (Oesch et al., 1985). The two prion protein isoforms differ in several respects: (a) PrP<sup>Sc</sup> possesses a protease-resistant core designated PrP 27-30; PrP<sup>C</sup> does not (Oesch et al., 1985). (b) Upon detergent extraction, PrP<sup>C</sup> is solubilized whereas PrP<sup>Sc</sup> forms macromolecular aggregates, and in the case of PrP 27-30, the aggregates assume a rod-shaped structure indistinguishable from many amyloids (Meyer et al., 1986). (c) PrP<sup>C</sup> is attached to membranes by a glycosyl phosphatidylinositol anchor at its carboxy terminus and can be released by digestion with phosphatidylinositol-specific phospholipase C (PIPLC) (Stahl et al., 1987). Although PrP<sup>Sc</sup> possesses a similar glycosyl phosphatidylinositol anchor, it is not releasable by PIPLC and its membrane topology is unknown (Stahl et al., 1990). (d) While both native and denatured PrP<sup>C</sup> are equally recognized by the presently available PrP antibodies, in many experimental situations PrP<sup>Sc</sup> is not immunoreactive unless it is first treated with protein-denaturing agents (Serban et al., 1990).

These differences in the properties of the two PrP isoforms appear to arise from an as yet unidentified posttranslational event (Basler et al., 1986; Borchelt et al., 1990).

Although PrP<sup>C</sup> is readily detected on the surface of normal and scrapie-infected cultured cells by immunofluorescence (Stahl et al., 1987), PrP<sup>Sc</sup> has remained intriguingly elusive. Selective removal of PrP<sup>C</sup> with PIPLC failed to reveal a scrapie-specific signal within infected cells.

All polyclonal and monoclonal antibodies raised, to date, against PrP 27-30 or synthetic PrP 27-30 peptides react equally well with both PrP isoforms on immunoblots after SDS-PAGE. However, we have recently found that these PrP antibodies react equally well with native and denatured PrP<sup>C</sup>, but relatively poorly with nondenatured PrP<sup>Sc</sup> after limited proteolysis catalyzed by proteinase K. After terminating the limited proteinase K digestion with PMSF, we found that PrP<sup>Sc</sup> immunoreactivity was greatly enhanced by exposure to protein denaturants such as chaotropic salts, NaOH, SDS (at 100°C) and formic acid (Serban et al., 1990), all of which are known to diminish prion titers (Millson et al., 1976; Millson and Manning, 1979; Prusiner et al., 1980, 1981; Bolton et al., 1984). In earlier studies, one of us reported that formic acid treatment of brain sections enhanced the PrP immunoreactivity of amyloid plaques in prion diseases (Kitamoto et al., 1987), while other investigators demonstrated that formic acid enhanced the PrP immunoreactivity of partially purified scrapie prion preparations in ELISA (Kasczak et al., 1987).

We now report the use of enhanced PrP<sup>Sc</sup> immunoreactivity after denaturation for the in situ detection of the scrapie prion protein isoform in fixed cells. We describe the identification of PrP<sup>Sc</sup>-producing colonies of cultured cells, and for the first time, the localization of PrP<sup>Sc</sup> within single cells. We report the isolation of scrapie-producing subclones of a novel Syrian hamster brain cell line (HaB). The PrP<sup>Sc</sup> content of these subclones correlates well with their infectivity. The ability of the HaB cells to support long-term PrP<sup>Sc</sup> accumulation appears to be temperature-sensitive.

We demonstrate that in both scrapie-infected hamster HaB (ScHaB) cells as well as mouse N<sub>2</sub>a (ScN<sub>2</sub>a) cells, PrP<sup>Sc</sup> accumulates primarily intracellularly in contrast to PrP<sup>C</sup> on the plasma membrane. By confocal microscopy, most of the

PrP<sup>Sc</sup> localized with intracellular WGA ligands, suggesting its association with the Golgi apparatus. In one ScHaB clone, PrP<sup>Sc</sup> also extensively localized with the Golgi protein MG-160 (Gonatas et al., 1989), while in another clone this colocalization was not observed. The nature of PrP<sup>Sc</sup> association with the Golgi apparatus remains to be studied. Equally important will be studies on the intracellular localization of PrP<sup>Sc</sup> (or PrP<sup>CJD</sup>) in human brains from patients dying of CJD and Gerstmann-Sträussler syndrome as well as animals with scrapie. The higher resolution achievable by immunoelectron microscopy may help resolve some of these issues.

## Materials and Methods

### Materials

All reagents for cell culture were obtained from the University of California San Francisco Cell Culture Facility. PrP antisera and the mAb 13A5 were prepared in our laboratory (Barry and Prusiner, 1986; Serban et al., 1990). The MG-160 antiserum was a generous gift from Dr. Nicholas Gonatas (University of Pennsylvania) (Gonatas et al., 1989). The secondary antibodies for the ELISA and the immunofluorescence were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Avidin coupled to Texas Red was bought from Cappel Laboratories (West Chester, PA). Biotinylated goat anti-rabbit IgG was purchased from Zymed Corp. (South San Francisco, CA). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). The Protoblot system for Western immunodetection was purchased from Promega Biotec (Madison, WI). Immobilon P was bought from Millipore Corp. (Bedford, MA). Nitrocellulose membranes for the colony assay were obtained from Schleicher & Schuell (Keene, NH). Proteinase K was purchased from Beckman Instruments Co. (Palo Alto, CA). The antifading agent *n*-propyl gallate was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). PIPLC was purified from *Bacillus thuringiensis* as described (Low et al., 1988).

### Cultured Cells

The isolation of scrapie-infected subclones of N<sub>2</sub>a cells has been described (Butler et al., 1988). The HaB cells were obtained from a primary culture of a scrapie-infected Syrian golden hamster (LVG/LAK; Charles River Laboratories, Wilmington, DE) brain as follows: the brain was removed 65 d after the intracerebral inoculation of 50  $\mu$ l of a 10-fold dilution of a scrapie brain homogenate ( $\sim 10^7$  ID<sub>50</sub> units). The organ was minced, trypsinized, and seeded onto 10 10-cm plates precoated with 0.5% bovine collagen (Sigma Chemical Co.) in DME-H21 medium supplemented with 10  $\mu$ g/ml insulin, 2 mM glutamine, 10% FCS, 5% heat-inactivated horse serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin and incubated at 37.5°C with 5% CO<sub>2</sub>. At confluence the cells were split at a ratio of 1:8. At the seventh passage, the cell division seemed to stop completely. A few plates were kept at 37.5°C for three additional months, at which time two continuously dividing cell populations appeared in two separate plates. They were designated HaB<sub>1</sub> and HaB<sub>2</sub>. PrP<sup>C</sup>, but no proteinase K-resistant PrP<sup>Sc</sup> was found in them. They grow in monolayers and are bipolar and sometimes tripolar in appearance. They were further grown at 37.5°C in DME-16 plus 10% FCS. The HaB<sub>1</sub> cells were used in the studies described here and have been designated HaB for convenience.

### Infection of HaB Cells

HaB cells ( $\sim 10^4$ ) were seeded in a single well of a 96-well microtiter plate. At confluence they were covered with purified hamster rods ( $\sim 10^6$  ID<sub>50</sub> units) in full medium and transferred to 34°C. 48 h later, the cells were washed, trypsinized and seeded in a larger plate at 1:5 dilution. The cloning described here was started after the eighth passage at 34°C. The cells were seeded in 96-well plates by limiting dilutions. Wells in which colonies developed were trypsinized and seeded in duplicates in new 96-well plates for the cell ELISA assay. For this study, all the clones were grown at 34°C.

### The Subclones ScHaB-4 and ScHaB-4-C4

Most of the work described here was performed with the persistently in-

ected clones ScHaB-4 and ScHaB-4-C4 of HaB. One of the PrP<sup>Sc</sup> positive clones obtained as described before (under Infection of HaB Cells) was designated ScHaB-4 (see Table II). This clonal cell line was passaged at 1:5 dilution approximately every 10 d at 34°C and examined from time to time for PrP<sup>Sc</sup> by immunofluorescence. After about a year in culture, the cells appeared to lose their PrP<sup>Sc</sup> content by this criterion. A PrP<sup>Sc</sup>-positive subclone of these cells, designated H3, was isolated at 34°C. This clone again appeared to lose its PrP<sup>Sc</sup> content after a few months of culture. A cloning attempt of these H3 cells, carried out at 37.5°C, yielded a new subclonal line, designated ScHaB-4-C4, which has been positive for PrP<sup>Sc</sup> for several months. This line was grown and passaged at 37.5°C.

### Antibodies

The rabbit polyclonal antisera R017 and R073 were raised against SDS-denatured purified rods and SDS-PAGE-purified PrP 27-30, respectively (Barry et al., 1985; Serban et al., 1990). They react with both hamster and mouse PrP.

The monoclonal antibodies 13A5 and 8C5 were obtained after immunization of mice with SDS-PAGE-purified hamster PrP 27-30 (Barry and Prusiner, 1986). These antibodies do not react with mouse PrP. 8C5 recognizes an epitope within the P5 region of the PrP gene (residues 140-174) and its activity can be adsorbed by preincubation with the synthetic peptide (10 µg/ml).

The rabbit antiserum R013 was raised against synthetic peptide PrP-P1 corresponding to PrP codons 90-102, which constitute the NH<sub>2</sub>-terminal segment of PrP 27-30. The synthetic peptide P1 (gly<sub>90</sub>-gln-gly-gly-thrhis-asn-gln-trp-asn-lys-pro-gly-gly-cys) was synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) as previously described (Barry et al., 1988). The residues in italics were added to facilitate coupling the peptide to carrier proteins when it was used as an immunogen.

### Prion Bioassays

Inocula from confluent cultures in 10-cm dishes (~10<sup>7</sup> cells) were prepared as follows: the cells were rinsed twice in cold PBS, scraped in PBS, pelleted, and resuspended in 800 µl of bioassay buffer (5% BSA in PBS). The suspension was frozen and thawed twice and then sonicated for 10 s in a bath sonicator. The disrupted cells were inoculated into four Syrian hamsters for each sample (50 µl per hamster) and the prion titers were calculated as previously described (Prusiner et al., 1982).

### PIPLC Digestion of Cells

In some instances cell monolayers were incubated with 3 U/ml PIPLC (Low and Finean, 1977; Stahl et al., 1987) in DME-16 plus 2% FCS at 37°C for 5 h.

### Preparation of Cell Lysates for Gel Electrophoresis

Cells in 10-cm culture dishes were lysed with 1 ml of ice-cold lysis buffer (0.5% NP-40, 0.5% Na-deoxycholate, 100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.8). Insoluble material was removed with a low-speed spin. When needed, the lysates were then incubated with 15 µg/ml proteinase K for 1 h at 37°C. The reaction was stopped by the addition of PMSF to 3 mM. After a 30-min incubation on ice, 1 vol of 2× SDS-sample buffer was added and the lysate was boiled for 5 min before its loading on the gel.

### SDS-PAGE and Western Blotting

Electrophoresis of proteins on 12% polyacrylamide gels was performed according to the method of Laemmli (1970). For Western analysis the proteins were electrotransferred (Towbin et al., 1979) to Immobilon paper. The membrane was blocked in 5% nonfat dry milk in TBST (0.05% Tween 20, 100 mM NaCl, 10 mM Tris, pH 7.8), incubated for 18 h with the primary antibody, and then stained with the Protoblot alkaline phosphatase system according to the manufacturer's instructions.

### Cell ELISA

The cells were grown in 96-well plates. At confluence they were washed twice with PBS, fixed for 30 min either in 4% formaldehyde (when the rabbit antisera were used as primary antibodies) or in 1.3% formaldehyde (for the mAbs) in PBS, then incubated with 0.1 M glycine in PBS for 1 min, and finally permeabilized with 0.4% Triton X-100 in PBS for 3 min at room

temperature. For proteinase K digestion the cells were incubated with 200 µl of 20 µg/ml proteinase K in PBS for 1 h at 37°C. The cells were then washed and treated with PMSF (3 mM in PBS for 30 min). For guanidine hydrochloride (GdnHCl) denaturation, 150 µl of 6 M GdnHCl, 50 mM Tris-HCl, pH 7.4 was added to each well (10 min, room temperature); the cells were then rinsed twice with PBS.

Before antibody incubation, the cells were blocked in 5% nonfat dry milk in PBS plus 5% nonfat milk. R013, R017, and R073 were used at dilutions 1:1,000, 1:1,000, and 1:2,000 in TBST, respectively, whereas the hybridoma media were used undiluted. The primary antibodies were incubated with the cells for 12-18 h at room temperature. The secondary antibody was usually peroxidase linked and *o*-phenylenediamine was used as the chromogen.

### Indirect Immunofluorescence

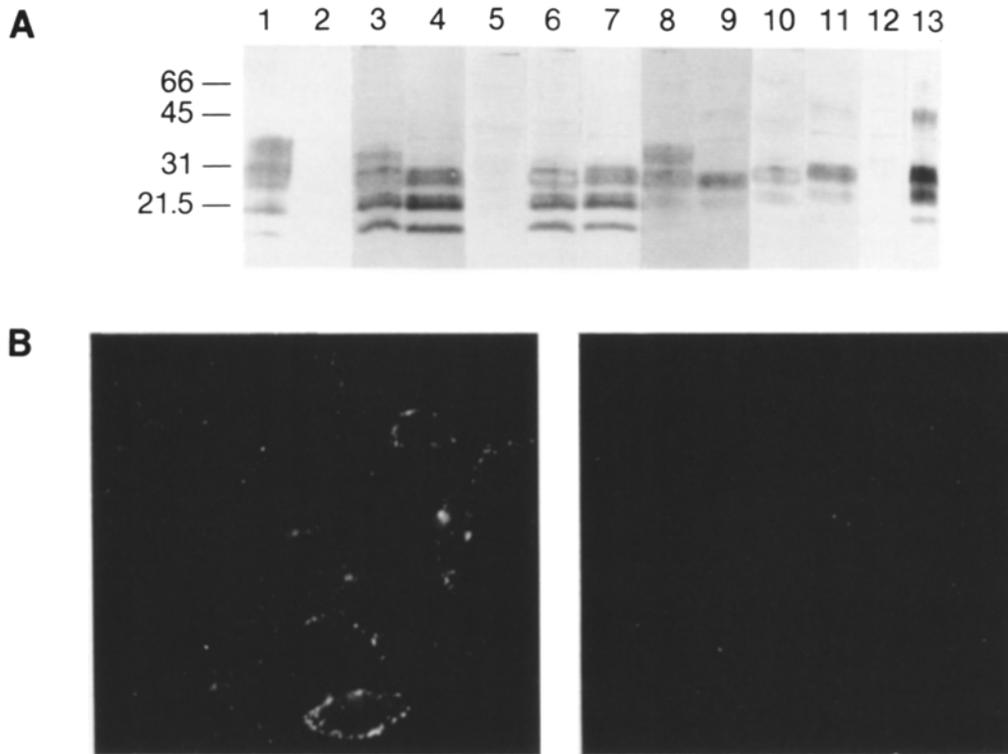
For immunofluorescent assays the cells were grown on eight-well slides. They were treated as described for the cell ELISA. Fluorescein-linked goat anti-rabbit IgG or goat anti-mouse IgG plus IgM were used at 1:50 dilution in PBS plus 5% nonfat milk (4 h, room temperature) and then the cells were extensively rinsed with PBS. For MG-160 detection, the cells were incubated for 12-18 h with the MG-160 antiserum diluted at 1:500 in PBS nonfat milk. They were then sequentially incubated with a biotinylated goat anti-rabbit IgG antibody (used undiluted as provided by the manufacturer) and with Texas Red-conjugated avidin (50 µg/ml in PBS and 1% BSA). The two final incubations were for 3 h each at room temperature. For WGA labeling, the cells were incubated with Texas Red-conjugated WGA (100 µg/ml in PBS and 1% BSA) for 10 min at room temperature. After mounting in 5% *n*-propyl gallate in 70% glycerol, pH 9.0 (Giloh and Sedat, 1982), the cells were examined by epifluorescence in a Leitz Orthomat microscope and photographed with Kodak 124 ASA film. For confocal microscopy, the samples were examined with a Lasersharp MRC500 system (Bio-Rad Laboratories).

### PrP<sup>Sc</sup> Colony Assay

Cells grown in 10-cm plates were rinsed twice with PBS and were then transferred onto nitrocellulose membranes supported by a circular filter paper. The membranes and filter papers were soaked in lysis buffer (100 mM NaCl, 10 mM Tris HCl 7.8, 10 mM EDTA, 0.5% NP-40, 0.5% Na deoxycholate) before their application on the cells. Several minutes after placing the nitrocellulose membrane backed by filter paper on the plate of cells, a dry filter paper was applied onto the wet filter to blot the excess liquid. The nitrocellulose membrane was then removed, thoroughly air dried, and rinsed in TBST. For proteinase K digestion, the membranes were incubated in 50-150 µg/ml proteinase K in TBST for up to 18 h at 37°C with constant rocking (Serban et al., 1990). The optimal proteinase K concentration is critical and batch dependent. For maximal sensitivity of the assay, the concentration should be adjusted so that no signal appears in normal cells upon immunoprobng. However, a detectable signal was obtained from scrapie-infected colonies even when the membranes were incubated with as much as five times the optimal proteinase K concentration. During the course of these studies, we found later that the 0.05% Tween 20 in TBST may be advantageously replaced by 0.1% Brij 35 in the protease buffer resulting in a reduction of background. After removal of the proteinase K, the nitrocellulose membrane was incubated in 3 mM PMSF at room temperature for 30 min followed, when needed, by a treatment with Tris-buffered 3 M guanidine thiocyanate (GdnSCN) (Serban et al., 1990) for 8 min at room temperature. The membranes were subsequently processed as described above for Western blots.

### Results

Having found that antibodies raised against denatured PrP 27-30 react weakly with proteinase K-digested PrP<sup>Sc</sup> immobilized on solid support, but react well with PrP<sup>Sc</sup> after protein denaturation (Serban et al., 1990), we evaluated this phenomenon in formaldehyde-fixed cells using both cell ELISA and immunofluorescence examination. N<sub>2</sub>a, ScN<sub>2</sub>a, HaB and two clones of ScHaB: ScHaB-4 and ScHaB-4-C4, were used for this purpose. ScN<sub>2</sub>a are a persistently infected subclone of the mouse neuroblastoma line N<sub>2</sub>a (Butler et al., 1988). These cells are flatter and appear larger than



**Figure 1.** PIPLC releases PrP<sup>C</sup> but not PrP<sup>Sc</sup> from cells. (A) N<sub>2</sub>a and ScN<sub>2</sub>a cells were incubated for 5 h in the presence (lanes 5–7 and 10–13) or absence (lanes 1–4, 8, and 9) of PIPLC (3 U/ml). They were then lysed, digested with proteinase K (lanes 2, 4, 7, 9, and 11–13), and their PrP content analyzed by SDS-PAGE and Western blotting using R073 (1:5,000) (lanes 1–11) or the hamster-specific mAb 13A5 (supernatant 1:40) (lanes 12 and 13) as primary antibodies. In lanes 8–11 and 13, N<sub>2</sub>a cells were incubated with purified hamster prions (MOI = ~500 ID<sub>50</sub>/cell) for 24 h before PIPLC digestion and fixation. Notice that both endogenous PrP<sup>Sc</sup> (lanes 4, 6, and 7) and exogenous PrP<sup>Sc</sup> (lanes 9–11 and 13) are resistant to proteinase K and PIPLC digestions. In ScN<sub>2</sub>a cells most PrP<sup>Sc</sup> is endogenously degraded to PrP 27–30, possibly by cellular proteases (lane 6). In lane 13 the PIPLC- and proteinase K-resistant PrP reacted with the hamster-specific mAb 13A5 and thus originated from the exogenous hamster prions. No similar signal was detected by 13A5 in N<sub>2</sub>a cells not incubated with hamster prions (lane 12). (B) In *a*, PrP immunofluorescence on unfixed, living HaB cells. In *b*, HaB cells were treated with PIPLC (3 U/ml) for 5 h before immunofluorescence. The cells were then rinsed and assayed for membrane-bound PrP by indirect immunofluorescence on living cells using R017 as primary antibody. The antigen aggregates under these conditions (Stahl et al., 1987).

the original N<sub>2</sub>a cells (Butler et al., 1988; Fig. 2). ScHaB-4 and ScHaB-4-C4 are persistently infected subclones of the new hamster brain line HaB. Both the scrapie-infected N<sub>2</sub>a and HaB lines produce readily detectable amounts of both PrP isoforms (Butler et al., 1988; Figs. 1 and 9).

#### **A Scrapie-specific PrP Epitope Revealed by Denaturation**

Cells grown in microtiter wells were fixed with formaldehyde and permeabilized with Triton X-100; in some cases, this treatment was followed by *in situ* digestion with proteinase K as well as exposure to 6 M GdnHCl. ELISA for PrP was then performed on the cells using R017 (a polyclonal immune serum raised in rabbit against SDS-denatured hamster prion rods), R073 (a rabbit antiserum raised against SDS-PAGE-purified hamster PrP 27–30), or their preimmune sera as primary antibodies followed by a peroxidase-linked secondary antibody (Table I, experiment A).

The ELISA protocol (column 1) gives values for fixed cells that were neither proteinase K digested nor GdnHCl denatured. All of the cells reacted strongly with the immune sera

under these conditions, but not with the preimmune sera. After limited proteinase K digestion, the PrP signal was much reduced regardless of the scrapie status of the cells (Table I, column 2); thus, this signal reflects the presence of protease-sensitive PrP. GdnHCl denaturation of the proteinase K-digested cells restored the immunoreactivity in the scrapie-infected cells but not in uninfected cells (column 3). This phenomenon was not detected with the preimmune sera (Table I, experiment A). The reappearance of the PrP immunoreactivity after protein denaturation in the proteinase K-treated scrapie cells mimicked our previous results with purified prion fractions and crude brain extracts (Serban et al., 1990) and we interpreted it as an indication for the presence of PrP<sup>Sc</sup> in the cells.

We observed that extensive proteinase K digestion of the fixed cells resulted in the loss of many cells from the plate. The phase-contrast micrographs of proteolyzed cells illustrate the structural damage caused by the proteolysis (compare Fig. 3 A with Fig. 2). Consequently, the absorbance signals obtained after the proteolysis originate from considerably fewer cells than were present in the original monolayer. For

Table 1. Cell ELISA Detection of the Cellular and Scrapie PrP Isoforms

Exp.	Cell line	Antibody	n	No PIPLC				+PIPLC				
				Proteinase K GdnHCl	-	+	+	-	+	+	-	
				1	2	3	4	5	6	7	8	
				ELISA (A492 × 10 <sup>3</sup> )								
A	N <sub>2</sub> a	PreR017	2	100 ± 2	60 ± 10	-20 ± 20	70 ± 2	ND				
		R017	2	1,350 ± 40	10 ± 3	-10 ± 14	1,340 ± 70	ND				
		PreR073	2	280 ± 10	260 ± 20	140 ± 10	160 ± 10	280 ± 40	210 ± 30	130 ± 10	190 ± 10	
		R073	6	1,930 ± 90	270 ± 2	280 ± 5	1,830 ± 20	920 ± 20	300 ± 10	300 ± 10	780 ± 20	
	ScN <sub>2</sub> a	PreR017	2	90 ± 7	60 ± 10	0 ± 10	60 ± 1	ND				
		R017	2	700 ± 40	20 ± 20	440 ± 4	590 ± 40	ND				
		PreR073	2	270 ± 40	270 ± 10	230 ± 10	170 ± 40	300 ± 20	240 ± 10	190 ± 10	180 ± 1	
		R073	6	1,680 ± 110	280 ± 4	420 ± 20	1,770 ± 70	510 ± 20	260 ± 4	430 ± 10	1,190 ± 50	
	HaB	PreR073	2	270 ± 30	260 ± 50	230 ± 10	150 ± 1	230 ± 40	290 ± 20	250 ± 1	180 ± 10	
		R073	4	1,220 ± 20	130 ± 10	150 ± 10	1,370 ± 30	520 ± 60	90 ± 10	120 ± 50	410 ± 40	
	ScHaB-4-C4	PreR073	2	230 ± 20	210 ± 0	200 ± 10	150 ± 20	240 ± 20	280 ± 10	220 ± 3	210 ± 10	
		R073	4	1,230 ± 100	290 ± 20	900 ± 60	1,020 ± 200	550 ± 50	230 ± 2	700 ± 30	830 ± 80	
B	ScN <sub>2</sub> a	R013	2	1,300 ± 30	90 ± 20	360 ± 20	ND	ND				
		R013 + P1	2	170 ± 10	4 ± 30	150 ± 20	ND	ND				
C	N <sub>2</sub> a + Rods	R017	2	>2,000	30 ± 10	700 ± 10	>2,000	ND				
		Mouse anti-hamster PrP monoclonal antibodies										
		13A5	4	80 ± 30	NA	NA	620 ± 20	60 ± 20	NA	NA	560 ± 40	
		8C5	4	60 ± 20	NA	NA	250 ± 10	50 ± 10	NA	NA	240 ± 20	
		8C5 + P5	4	60 ± 30	NA	NA	110 ± 10	60 ± 20	NA	NA	90 ± 6	

Normal (N<sub>2</sub>a and HaB) or scrapie-infected (ScN<sub>2</sub>a and ScHaB-4-C4) cells were seeded in 96-well microtiter plates. At confluence they were fixed, and in some cases, they were then sequentially incubated with proteinase K and GdnHCl as indicated. An ELISA was then performed on the cells using the indicated primary antibodies. In lanes 5–8, the cells were digested with PIPLC before their fixation. Experiment A: Protein denaturation exposes a scrapie-specific PrP epitope that is resistant to PIPLC and proteinase K digestions. The immune ELISA readings strongly decreased following proteinase K digestion of the cells or their incubation with PIPLC (lanes 2, 5, and 6) regardless of their scrapie status, but was partially restored in the scrapie-infected cells after the protein-denaturing step (lanes 3, 7 and 8). Rabbit antisera R017 and R073 were raised against denatured hamster prion rods and SDS-purified PrP 27–30, respectively; preR017 and preR073 are their respective preimmune sera. Experiment B: The guanidine-dependent signal in ScN<sub>2</sub>a cells is PrP specific. Preincubation of the PrP peptide P1 antiserum R013 with the synthetic oligopeptide P1 inhibited the GdnHCl-dependent signal (lane 3). Experiment C: GdnHCl exposes a PrP-specific epitope present on exogenous hamster PrP<sup>Sc</sup> internalized by N<sub>2</sub>a cells. Confluent N<sub>2</sub>a cells were incubated in situ with hamster rods (~5 × 10<sup>2</sup> ID<sub>50</sub> units/cell) for 24 h, then rinsed thoroughly, fixed and processed as above. 13A5 and 8C5 are hamster PrP-specific mAbs. Incubation of 8C5 with the PrP peptide P5 inhibits its binding (see Materials and Methods). The numerical values represent the intensity of the color reaction in optical density units at 492 nm. NA, not applicable. For the mAb, the cells were less well fixed (1.3% formaldehyde, 30 min) and could not withstand the proteinase K digestion. n, the number of wells used to generate the data.

this reason, the signal obtained after the proteinase K digestion followed by GdnHCl denaturation should be considered as a qualitative indicator of PrP<sup>Sc</sup> but not as a quantitative measure of PrP<sup>Sc</sup> concentration.

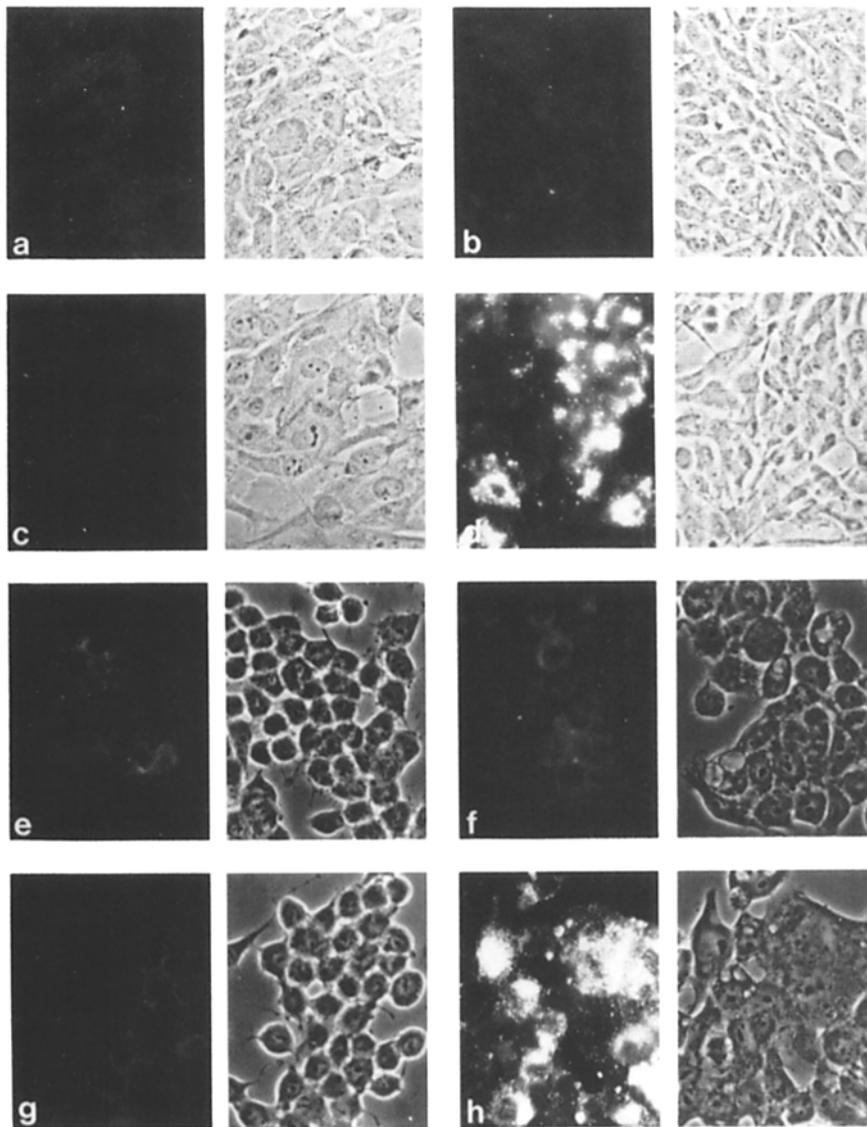
The enzyme PIPLC has been shown to release PrP<sup>C</sup>, but not PrP<sup>Sc</sup> from cultured cells (Stahl et al., 1987, 1990). Thus, PIPLC provides a tool to verify that the proteinase K, GdnHCl signals detected by ELISA are specific for PrP<sup>Sc</sup>. We confirmed the action of PIPLC on N<sub>2</sub>a and ScN<sub>2</sub>a cells by analyzing their PrP content with and without PIPLC digestion. The cells were digested with the enzyme for 5 h and lysed; their PrP was analyzed by Western blotting. As seen in Fig. 1 A, the enzymatic digestion reduced strongly the PrP<sup>C</sup> signal in both cell lines but not the proteinase K-resistant PrP<sup>Sc</sup> signal in ScN<sub>2</sub>a cells, as expected (compare lanes 1 and 5 [N<sub>2</sub>a] and 3 and 6 [ScN<sub>2</sub>a]). The action of PIPLC was also visualized by immunofluorescence performed on living HaB cells (Fig. 1 B).

When the cells were digested with PIPLC before fixation for the ELISA assay, the PrP<sup>C</sup> signal was strongly reduced (compare columns 5 and 1 of Table I, experiment A),

whereas no significant change was noted in the guanidine-dependent signal of the proteinase K-digested, scrapie-infected cells, as expected for PrP<sup>Sc</sup> (Table I, columns 3 and 7). The vast reduction of the PrP<sup>C</sup> background by the PIPLC digestion alone, without further proteolysis, allowed us to uncouple the effects of the guanidine from those of the proteinase K. As seen in Table I, experiment A, lanes 5 and 8, a guanidine incubation of the fixed cells again strongly increased the ELISA signals in the infected cells, but did not have this effect in the uninfected cells.

To confirm the PrP specificity of the cell ELISA results, we repeated the experiments with R013, a monospecific antiserum raised in rabbit against the PrP peptide P1 (Barry et al., 1988). Results similar to those obtained with the polyspecific antisera R017 and R073 were observed (Table I, experiment B). Furthermore, the synthetic peptide P1 was found to be effective in absorbing the immunoreactivity, demonstrating the PrP specificity of the signals (Table I, experiment B).

To verify further that the GdnHCl-dependent signal indeed correlates with the scrapie isoform PrP<sup>Sc</sup>, we assessed the

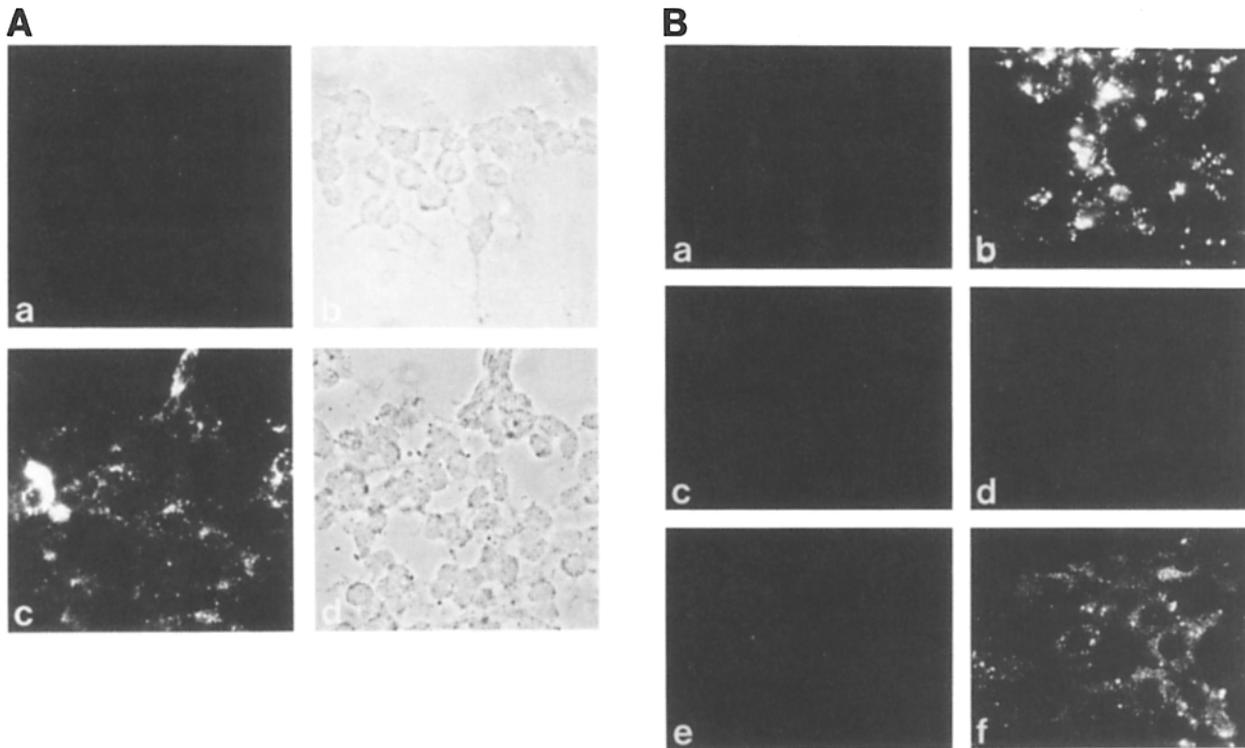


**Figure 2.** Guanidine treatment is essential for the immunofluorescent detection of PrP<sup>Sc</sup> in ScHaB-4-C4 and ScN<sub>2</sub>a cells. HaB (*a* and *c*), ScHaB-4-C4 (*b* and *d*), N<sub>2</sub>a (*e* and *g*) and ScN<sub>2</sub>a (*f* and *h*) cells growing on glass slides were fixed with 4% formaldehyde for 30 min and permeabilized with 0.4% Triton X-100. In some panels, the cells were then treated with 6 M GdnHCl (*c*, *d*, *g*, and *h*). An immunofluorescent assay for PrP was then performed with R073 as the primary antiserum (1:2,000). Note the appearance of a speckled intracellular signal in the GdnHCl-treated, scrapie-producing ScHaB-4 (*d*) and ScN<sub>2</sub>a (*h*). This signal could not be revealed when the guanidine step was omitted (*a*, *b*, *e*, and *f*) even if harsher permeabilization procedures were used (10% Triton X-100 or 2% Sarkosyl [data not shown]). These results indicate that protein denaturation is needed to expose the epitopes of the intracellular, speckled PrP accumulations. Phase-contrast micrographs are presented together with the corresponding fluorescence pictures. Extreme care was exercised to keep all photographic exposures and processing times equal in each set of fluorescent micrographs (HaB cells, *a*-*d*; N<sub>2</sub>a cells, *e*-*h*).

ability of the ELISA to detect exogenous prions internalized in cells. When N<sub>2</sub>a or HaB cells are incubated with purified hamster prion rods, some PrP<sup>Sc</sup> is internalized. This was confirmed by a Western blot analysis of N<sub>2</sub>a cells (Fig. 1 *A*, lanes 8-13). The cells were incubated for 24 h in the presence of hamster prion rods (multiplicity of infection [MOI] = ~500 ID<sub>50</sub>/cell), rinsed twice with PBS, and then incubated for 5 h in medium with or without PIPLC. The cells were then lysed and their PrP content was analyzed by SDS-PAGE followed by Western blotting. The presence of proteinase K-resistant PrP 27-30 species in those cells is demonstrated in lanes 9-11 and 13. These species were resistant to PIPLC (lanes 10, 11, and 13). Their detection with the hamster-specific mAb 13A5 confirms their exogenous origin (lane 13). These N<sub>2</sub>a cell-associated rods could also be visualized by immunofluorescence and similar results were obtained for HaB cells incubated with prion rods (see Fig. 3 *B*).

N<sub>2</sub>a and HaB cells were thus incubated with prion rods as described above, washed, and in some cases, treated with PIPLC. The cells were then rinsed and subjected to the PrP

cell ELISA (Table I, experiment C). As shown, GdnHCl denaturation increased the PrP immunoreactivity in the proteinase K-treated cells as well as in the cells digested with PIPLC, similar to the phenomenon found with scrapie-infected cells. We again checked that this GdnHCl-dependent PrP signal can be detected with the hamster-specific mAbs 8C5 and 13A5. 8C5 recognizes an epitope within the region of the PrP sequence bounded by residues 140-174. The immunoreactivity of 8C5 can be adsorbed by the synthetic peptide P5 that corresponds to these residues. As the epitopes recognized by these antibodies are very sensitive to formaldehyde fixation, their use required a weaker fixation (1.3% formaldehyde, 30 min). We found that the cells fixed under those conditions do not withstand the proteinase K digestion. We thus omitted the proteolysis when using 13A5 and 8C5 and relied on the PIPLC to reduce the amount of cellular PrP. As shown in Table I, experiment C, both mAbs recognized GdnHCl-dependent, PIPLC insensitive hamster PrP epitopes in fixed N<sub>2</sub>a cells preincubated with hamster prions, but not in uninoculated cells (data not shown). More-



**Figure 3.** The guanidine-dependent PrP signal in ScHaB-4-C4 cells is resistant to sequential PIPLC and proteinase K digestions. (A) ScHaB-4-C4 cells were digested with PIPLC (3 U/ml for 5 h at 37°C), then fixed with 4% formaldehyde (room temperature, 30 min) and digested with proteinase K (20 µg/ml, 37°C, 1 h). They were then processed for PrP immunofluorescence with (c) or without (a) GdnHCl treatment, using R073 (1:2,000), as primary antibody. b and d are the corresponding phase-contrast micrographs. Note the extensive damage to the cells caused by the proteolysis. (B) Internalization of hamster prions by N<sub>2</sub>a and HaB cells revealed by guanidine-PrP immunofluorescence. N<sub>2</sub>a (a, b, c, and d) and HaB cells (e and f) were incubated with purified hamster prion rods (MOI = ~500 ID<sub>50</sub>/cell) for 24 h. They were then washed, digested with PIPLC (3 U/ml for 5 h at 37°C), fixed with 1.3% formaldehyde, treated with GdnHCl (b, d, and f) and assayed for PrP using the hamster-specific mAb 8C5. In c and d, preincubation of the antibody with the synthetic PrP-peptide P5 adsorbed its activity and abolished the guanidine-dependent signal in d.

over, the Gdn-dependent signal obtained with 8C5 was strongly reduced when the antibody was preincubated with the synthetic peptide P5. We conclude that this hamster-specific PrP epitope is a consequence of the exogenous PrP<sup>Sc</sup> internalized by the cells.

The cell ELISA described above thus appears to detect both the cellular and scrapie PrP isoforms in fixed cells and to differentiate between them.

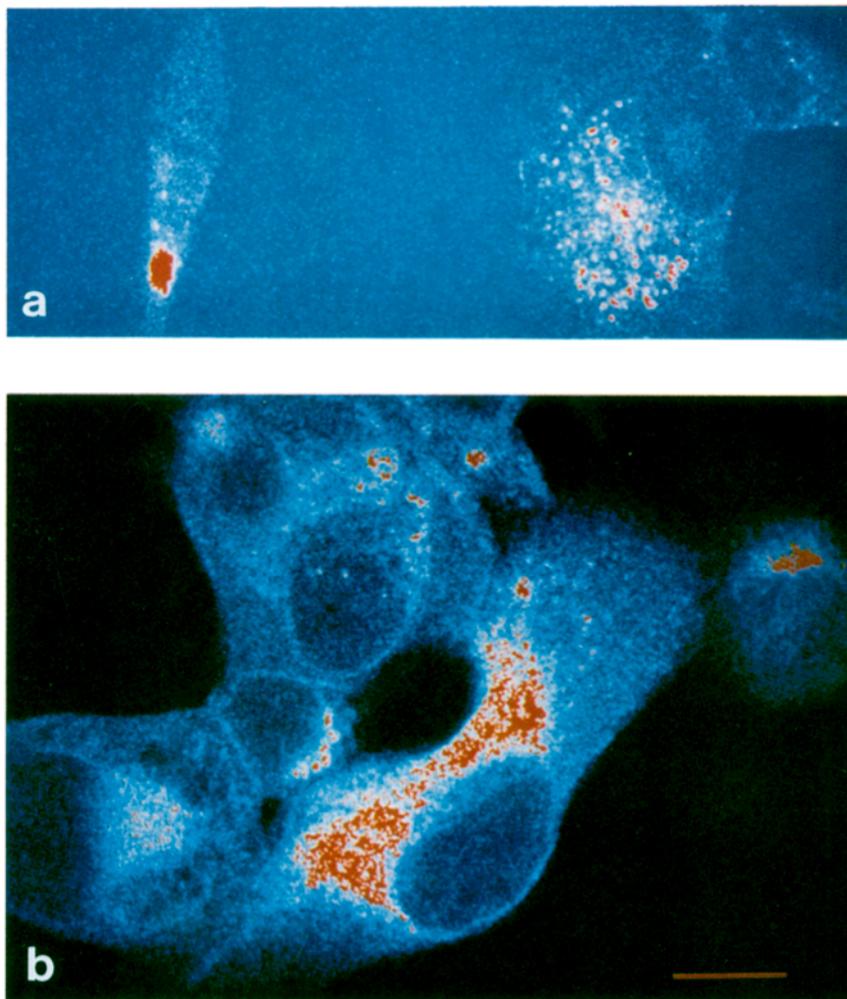
#### **Immunofluorescent Detection of PrP<sup>Sc</sup> Requires Denaturation**

Although plasma membrane-bound PrP<sup>C</sup> was detected by immunofluorescence (Stahl et al., 1987), attempts to detect PrP<sup>Sc</sup> by immunofluorescence in cultured cells were unsuccessful before the results reported here. Based on results from immunoblot studies (Serban et al., 1990) and the ELISA data in Table I, we reasoned that immunofluorescent detection of PrP<sup>Sc</sup> may require denaturation of PrP<sup>Sc</sup> molecules to enhance the binding of PrP antibodies. To explore this possibility, normal and scrapie-infected cells were seeded on slides, fixed, permeabilized, treated in some cases with GdnHCl, and assayed for PrP by indirect immunofluorescence, using R073 as the primary antibody.

An intense, intracellular signal appeared in GdnHCl-treat-

ed scrapie-infected ScHaB-4-C4 cells (Fig. 2 d) and ScN<sub>2</sub>a (h); no such signal was detected in either scrapie-infected cells untreated with GdnHCl (b and f) or in uninfected cells (a, c, e, and g). Similar results were obtained in both ScN<sub>2</sub>a and ScHaB cells when R017 (not shown) and the P1 peptide antiserum R013 (see Fig. 5) were used as the primary antisera, and in ScHaB cells with the mAbs 13A5 (see Figs. 5, 7, and 8) and 8C5 (not shown). The guanidine-dependent intracellular fluorescent signal in the infected cells was not detected with (a) preimmune sera, (b) omission of the primary antisera, or (c) omission of the secondary antisera (data not shown). The intracellular PrP signal was preserved when the cells were digested with PIPLC, fixed with formaldehyde, and then digested with proteinase K before immunofluorescence using the conditions developed for the cell ELISA (Table I; Fig. 3 A). As shown in the corresponding phase-contrast micrographs (Fig. 3 A, b and d), the extensive proteolysis compromised the structural integrity of the cells and therefore it was not routinely used. The guanidine-dependent PrP signal in the scrapie-infected cells was thus resistant to both PIPLC and proteinase K digestions, as expected for PrP<sup>Sc</sup>.

We assessed the need for a guanidine denaturation to reveal exogenous prions internalized by N<sub>2</sub>a and HaB cells. The cells were incubated with purified hamster prion rods



**Figure 4.** Immunofluorescent detection of PrP<sup>Sc</sup> in GdnHCl-treated ScHaB-4 and ScN<sub>2</sub>a cells by confocal microscopy. ScHaB-4 (a) and ScN<sub>2</sub>a (b) cells grown on glass slides were assayed for PrP immunofluorescence as indicated in the legend to Fig. 2. They were then examined by confocal microscopy. Bar, 10  $\mu$ m.

for 24 h, rinsed, treated with PIPLC for 5 h, and then prepared for PrP indirect immunofluorescence using 8C5, an mAb raised in mouse against SDS-PAGE-purified hamster PrP 27–30 (Barry et al., 1988). We used this hamster-specific mAb here because its activity can be adsorbed by the synthetic peptide P5. As shown in Fig. 3 B b, a series of bright internal dots appeared in the “infected” mouse N<sub>2</sub>a cells after guanidine denaturation, and this signal was abolished by preincubating the antibody with the synthetic peptide P5 (d). No similar signal appeared in guanidine-treated N<sub>2</sub>a cells not previously incubated with prion rods (not shown). The exogenous rods could not be detected if the cells were not treated with guanidine (Fig. 3 B, a and c). We again conclude that guanidine denaturation is essential to permit the immunofluorescent detection of exogenous PrP<sup>Sc</sup> using this antibody. Similar results were obtained for prions internalized in HaB cells using the mAb 13A5 (Fig. 3 B, e and f), and with the polyclonal antibodies R017 and R073 (not shown).

The need for GdnHCl denaturation for detection of PrP<sup>Sc</sup> is illustrated for both ScHaB cells and ScN<sub>2</sub>a cells (Figs. 2 and 3). Omission of the GdnHCl denaturation step prevented immunostaining of PrP<sup>Sc</sup> within the cell, although 0.4% Triton X-100 was routinely used to permeabilize the cells

and thus permit entry of the antibody. Identical results were obtained when a higher concentration of detergent was used in the permeabilization (up to 10% Triton X-100 or 2% Sarkosyl [sodium lauryl sarcosinate]), arguing that the absence of intracellular signals in non-GdnHCl-treated cells was not due to membrane impermeability (data not shown).

#### Cellular and Scrapie PrP Isoforms

The foregoing results show that the guanidine-dependent intracellular signal appearing in denatured ScHaB-4 and ScN<sub>2</sub>a cells represents a proteinase K- and PIPLC-resistant PrP species, appearing only in scrapie-infected cells. Based on the known biochemical properties of the PrP isoforms, we argue that these PrP accumulations are composed mainly, if not solely, of PrP<sup>Sc</sup>.

In the cell ELISA, removal of PrP<sup>C</sup> was mandatory for the specific detection of PrP<sup>Sc</sup>. This was accomplished by either limited proteinase K or PIPLC digestion (Table I). In early studies (Stahl et al., 1987) intense, patchy surface fluorescence of cultured cells was demonstrated by addition of PrP antibodies to living cells. These antibodies induced the patching of PrP<sup>C</sup>, a phenomenon recorded for many other cell surface proteins (Fig. 1 B). In contrast, fixation

of the cells before immunostaining prevented patching of PrP<sup>C</sup> (Fig. 2). Because the immunofluorescence method developed for PrP<sup>Sc</sup> detection requires fixation of the cells before immunostaining, signals due to PrP<sup>C</sup> were minimal. Under these conditions, PrP<sup>C</sup> is detected as a diffuse fluorescence on the cell membrane and hence does not interfere with the intracellular PrP<sup>Sc</sup> signal (Fig. 2, *d* and *h*). Thus, in contrast with the cell ELISA assay, PrP<sup>Sc</sup> can be specifically detected by immunofluorescence without prior removal of PrP<sup>C</sup> by proteinase K or PIPLC.

### Confocal Microscopy

The subcellular distribution of PrP<sup>Sc</sup> was more precisely observed by confocal microscopy (White et al., 1987) of cells labeled by immunofluorescence after GdnHCl treatment (Fig. 4). As expected, PrP<sup>Sc</sup> was found primarily within the intracellular space although weak fluorescent signals were observed on the plasma membrane.

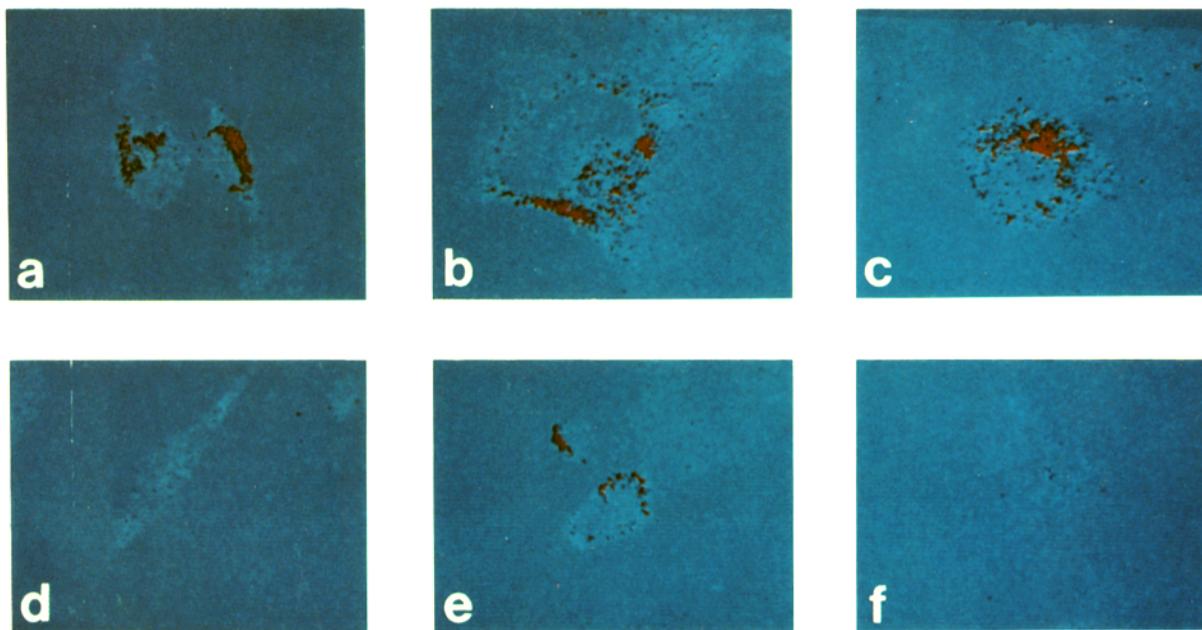
The scrapie-specific intracellular signal was also detectable in ScHaB-4 cells with the hamster-specific mAb 13A5 (Fig. 5, *b* and *e*) and in both ScHaB-4 and ScN<sub>2</sub>a cells with a monospecific antiserum (R013) raised in a rabbit against the NH<sub>2</sub>-terminal peptide of PrP 27–30 designated P1 (*a* and *c*). In the latter case, the GdnHCl-dependent intracellular signal could be prevented completely by preincubating the antibody with the synthetic oligopeptide P1 (Fig. 5, *d* and *f*) thereby demonstrating its PrP-specificity. In additional experiments an identical guanidine-dependent intracellular signal was observed in ScHaB-4 cells using 8C5. Again, the signal obtained with this antibody could be completely abol-

ished by preincubating the antibody with the free peptide P5 (data not shown). These results further confirm the PrP specificity of the intracellular antigen detected in the scrapie-infected cells.

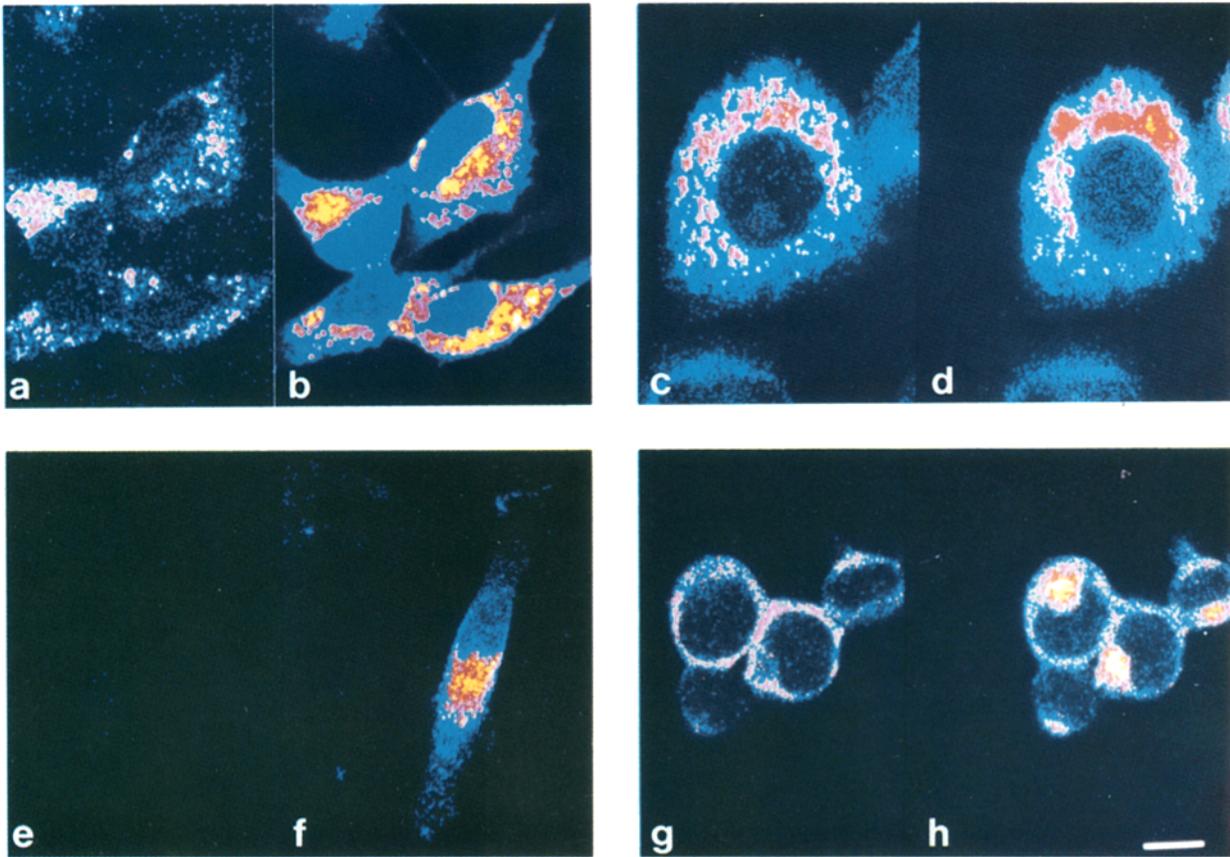
### Subcellular Localization of PrP<sup>Sc</sup>

When the PrP<sup>Sc</sup> immunostaining was compared to the intracellular pattern of WGA binding considerable overlap was observed (Fig. 6). The WGA signal was diminished when 0.4 M *N*-acetylglucosamine was included during the lectin incubation with the cells, confirming its specificity (not shown). WGA binds to clustered terminal *N*-acetylneuraminic acid residues as well as *N*-acetylglucosamine-containing oligosaccharides on proteins (Bhavanandan and Katlic, 1979) and labels cisternae along the distal face of Golgi, the *trans*-Golgi network, and the cell surface (Virtanen et al., 1980; Tarkoff and Vassali, 1983).

To extend these studies, a rabbit polyclonal antiserum raised against the rat brain mid-Golgi protein MG-160 (Gonatas et al., 1989) was compared with PrP<sup>Sc</sup> immunostaining in ScHaB-4 cells. As shown in Fig. 7, the MG-160 antiserum and the PrP mAb stained many of the same structures. A similar partial colocalization was observed in every ScHaB-4 cell examined. More than 30 cells were examined for PrP<sup>Sc</sup> localization. However, we repeated the double staining experiment with the ScHaB-4-C4 subclone when it became available, and found that in these cells PrP<sup>Sc</sup> and MG-160 did not completely colocalize (Fig. 8). More precise subcellular localization of PrP<sup>Sc</sup> will require immunoelectron microscopic studies.



**Figure 5.** The guanidine-dependent intracellular signal in ScHaB-4 and ScN<sub>2</sub>a cells is PrP specific. The PrP content of fixed, GdnHCl-treated ScHaB-4 (*a*, *b*, *d*, and *e*) or ScN<sub>2</sub>a (*c* and *f*) cells was analyzed by indirect immunofluorescence as indicated in the legend to Fig. 2, using the mAb 13A5 (undiluted supernatant) (*b* and *e*) or the monospecific anti PrP-P1 antibody R013 (1:1,000) (*a*, *c*, *d*, and *f*) as primary antibodies. In *d*, *e*, and *f* the antibodies were incubated with the synthetic oligopeptide P1 (10 μg/ml) before their use. The complete disappearance of the intracellular signal in *d* and *f* confirms its PrP identity.



**Figure 6.** PrP<sup>Sc</sup> and WGA partially colocalize in ScN<sub>2</sub>a and ScHaB-4 cells. ScHaB-4 (*a* and *b*), HaB (*e* and *f*), ScN<sub>2</sub>a (*c* and *d*) and N<sub>2</sub>a (*g* and *h*) cells grown on glass slides were processed for PrP<sup>Sc</sup> immunodetection using R073 (1:2,000) as primary antibody and a fluorescein-conjugated secondary antibody and then incubated with Texas Red-conjugated WGA (100 μg/ml). The fluorescent patterns in the fluorescein channel (PrP, *a*, *c*, *e*, and *g*) and the Texas Red channel (WGA, *b*, *d*, *f*, and *h*) were observed by confocal microscopy. ScN<sub>2</sub>a cells are flatter and appear larger than the noninfected N<sub>2</sub>a cells (see also Fig. 2, *e* and *f*; and Butler et al., 1988). Bar, 10 μm.

#### **Optimal Conditions for PrP<sup>Sc</sup> Accumulation in HaB Cells Defined by a Colony Assay for PrP<sup>Sc</sup>**

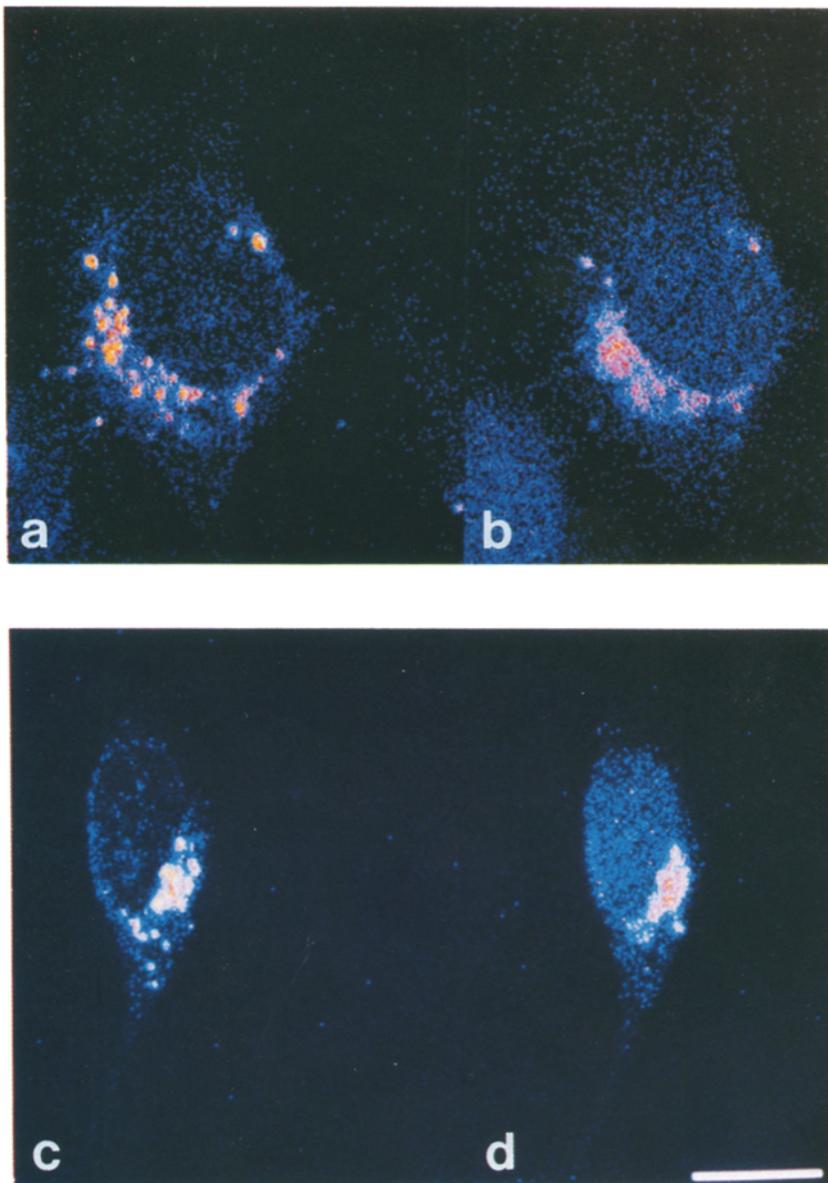
We took advantage of the convenience and sensitivity of the cell ELISA to screen for PrP<sup>Sc</sup>-containing subclones of ScHaB cells. HaB arose as a spontaneously immortalized cell population in a culture of a dissociated scrapie-infected hamster brain (see Materials and Methods). Lysates of the original population contained PrP<sup>C</sup> but not PrP<sup>Sc</sup> (Fig. 9, lanes 1 and 2) and no scrapie infectivity by bioassay (Table I, B). We attempted to infect the HaB cells by incubating them with purified hamster scrapie prion rods (see Materials and Methods). The cells were then cloned by limiting dilution and the clones assayed for PrP<sup>Sc</sup> by cell ELISA.

In early attempts to infect HaB cells, they were held at 37.5°C and no PrP<sup>Sc</sup>-producing clones were detected (data not shown). In contrast, when the cells were maintained at 34°C after their infection, ~15% of the clones produced PrP<sup>Sc</sup> and scrapie infectivity, as discussed below. The success of the infection appears to depend upon the temperature at which the cells are grown. This temperature dependence was observed on many occasions during the course of our study. To define optimal conditions for PrP<sup>Sc</sup> accumulation, we devised a colony assay for PrP<sup>Sc</sup>. Cells grown in a cell

culture dish were lysed and transferred in situ onto a nitrocellulose membrane. The PrP<sup>Sc</sup> was then measured after limited protease digestion and GdnSCN denaturation.

The colony assay for PrP<sup>Sc</sup> is illustrated in Fig. 10, *a-c* where normal or scrapie-infected N<sub>2</sub>a or HaB cells seeded in recognizable patterns on a dish were assessed for PrP<sup>Sc</sup>. The subclone of ScHaB cells designated ScHaB-4 was used as a positive control; its isolation is described below. After proteinase K digestion and GdnHCl treatment, only the ScN<sub>2</sub>a and ScHaB-4 cells were recognized by the polyclonal antibody R073 (Fig. 10 *b*), whereas the hamster-specific mAb 13A5 bound solely to the ScHaB-4 cells, as expected (Fig. 10 *c*). Noninfected cells did not react with the PrP antibodies after proteolysis and protein denaturation (Fig. 10, *b* and *c*).

To analyze the temperature sensitivity of PrP<sup>Sc</sup> synthesis in HaB cells, de novo infected ScHaB cells (see Materials and Methods) were seeded at low density in 10-cm dishes, grown at either 37.5°C (Fig. 2, *d-f*) or 34°C (Fig. 10, *g-i*) and analyzed for PrP<sup>Sc</sup> after the appearance of sizable colonies. Whereas a few hundred colonies grew at both temperatures, only 3 of them grown at 37.5°C proved to contain PrP<sup>Sc</sup> (*f*) in contrast to almost 100 grown at the lower temperature (*i*).



**Figure 7.** PrP<sup>Sc</sup> and the mid-Golgi protein MG-160 partially colocalize in ScHaB-4 cells. ScHaB-4 cells were fixed, permeabilized, and treated with GdnHCl. They were then simultaneously labeled with the PrP mAb 13A5 (undiluted supernatant) (fluorescein channel, *a* and *c*) and MG-160 (1:500) antiserum (Texas Red channel, *b* and *d*). Bar, 10  $\mu$ m.

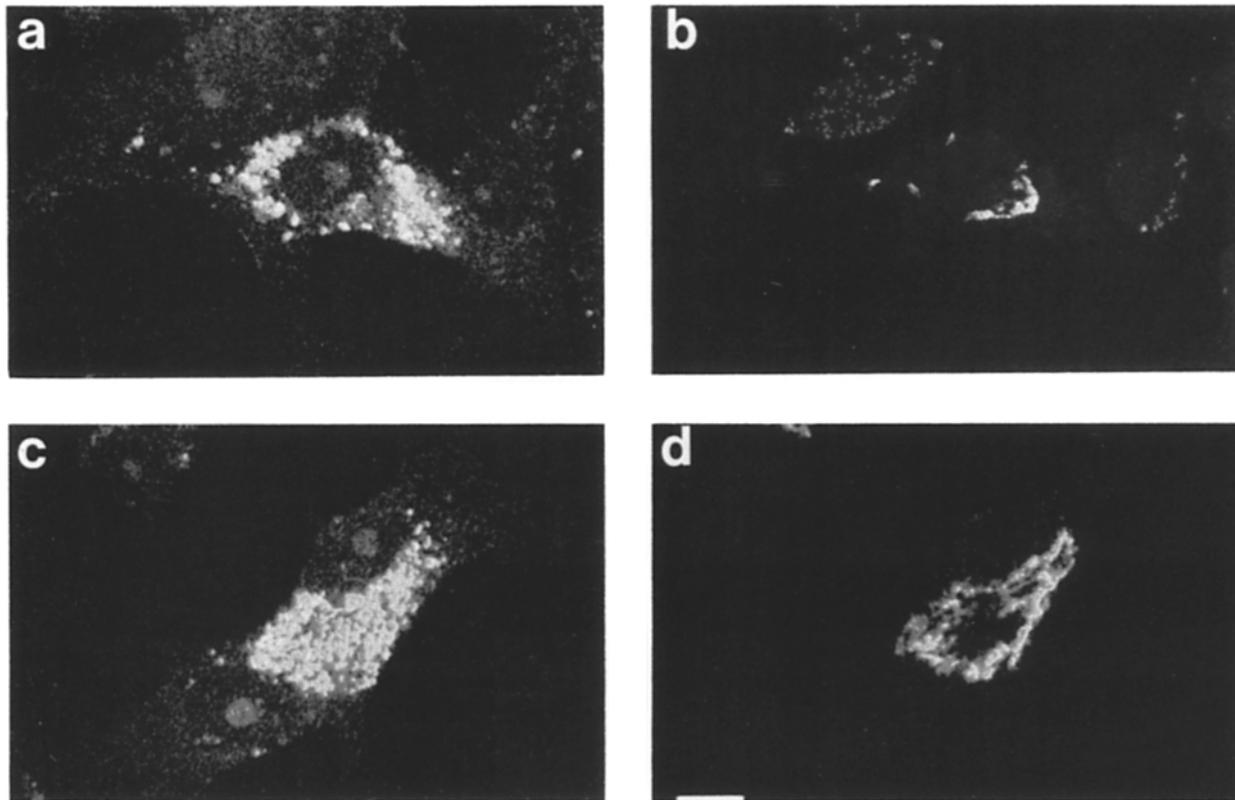
The reason for the apparent temperature sensitivity of PrP<sup>Sc</sup> production in ScHaB cells is still unknown. One possibility is that at the higher temperature, the cell division rate exceeds the rate of PrP<sup>Sc</sup> synthesis, so that PrP<sup>Sc</sup> is diluted out and eventually disappears from the culture. This paradigm assumes that PrP<sup>Sc</sup> is in some way involved in the appearance of new PrP<sup>Sc</sup> molecules.

#### **Isolation of ScHaB Clones**

Based on the foregoing results, we performed the infection of the HaB cells and the subsequent cloning at 34°C. Eight passages after infection, the cells were cloned by limiting dilution. Of 96 clones analyzed for PrP<sup>Sc</sup> by ELISA, 14 displayed a marked increase in their immunoreactivity with the PrP 27–30 antiserum R017 after GdnHCl denaturation of proteinase K-digested cells (not shown), indicating that they contained PrP<sup>Sc</sup>. After three further passages, the positive

clones (1–14) and nine negative clones (15–23) were examined again by ELISA. All the positive clones retained PrP<sup>Sc</sup> except number 7 (Table II). There was an excellent correlation between the PrP<sup>Sc</sup> signal and the infectivity of subclones. The de novo infected cells were passaged eight times at a 1:5 dilution before their cloning. The clones were subsequently expanded by a factor of  $\sim 10^8$  (to 10 10-cm dishes) before their infectivity was assayed. The overall expansion of these cells between the time of their infection and the bioassays was thus  $>10^{11}$ , ruling out the possibility that their measured infectivity was left from the inoculum used in the infection. We also confirmed the presence of PrP<sup>Sc</sup> in the ELISA positive HaB clones by Western blotting as illustrated in Fig. 9, lanes 3–7. No cytopathology within the scrapie-infected cells was observed by phase-contrast microscopy (see Fig. 2).

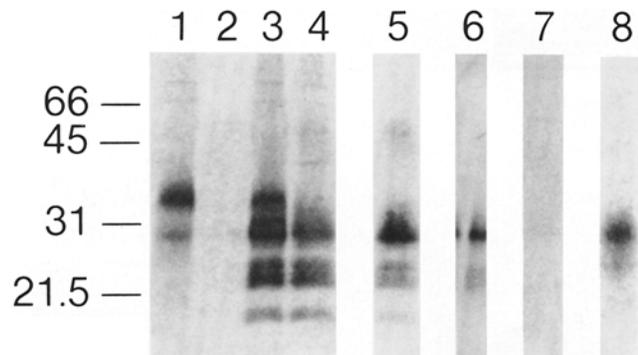
The excellent correlation between PrP<sup>Sc</sup> and infectivity in the ScHaB-4 subclones was similar to that observed for



**Figure 8.** PrP<sup>Sc</sup> and MG-160 do not colocalize in the subclone ScHaB-4-C4. The cells were treated for PrP<sup>Sc</sup> (fluorescein channel, *a* and *c*) and MG-160 (Texas red channel, *b* and *d*) immunolabeling as indicated in the legend to Fig. 7.

ScN<sub>2</sub>a cells (Butler et al., 1988). These correlations argue persuasively that as in animals and humans (Prusiner, 1989; Serban et al., 1990) PrP<sup>Sc</sup> production accompanies prion infection in cultured cells.

As the colony assay indicated that stable infection of HaB cells at 37.5°C is possible, albeit rare (see Fig. 10*f*), we subsequently subcloned the ScHaB-4 line at that temperature and isolated one subclone positive for PrP<sup>Sc</sup> (see Materials and Methods). This subclone was designated ScHaB-4-C4 and some of its unique properties were described above (Fig. 8).



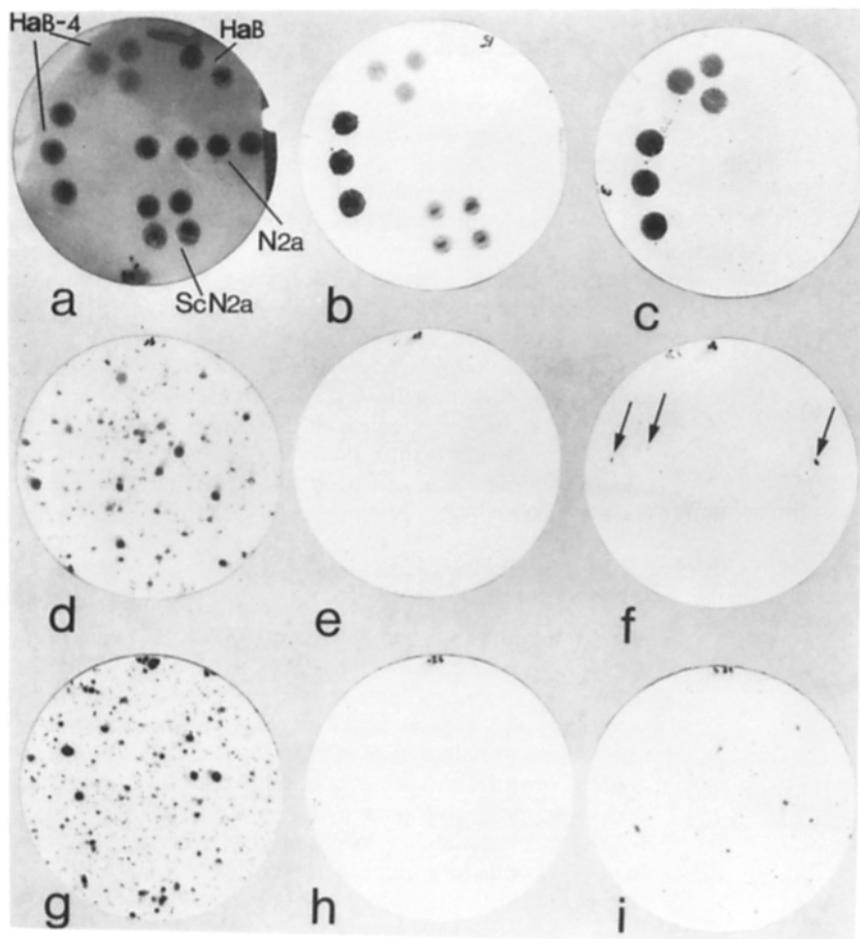
**Figure 9.** The infectious ScHaB-4 cells produce both PrP<sup>C</sup> and PrP<sup>Sc</sup>. Extracts from noninfected HaB (lanes 1 and 2) or persistently infected ScHaB-4 (lanes 3-7) cells were analyzed by SDS-PAGE. In lanes 2 and 4-7 the extracts were digested with 10 µg/ml proteinase K for 30 min at 37°C before the electrophoresis. A proteinase K-digested hamster scrapie-brain extract was added as a marker in lane 8. After transfer to an Immobilon membrane the proteins were probed with R073 (1:5,000) (lanes 1-4 and 8), the mAb 13A5 (supernatant 1:40) (lane 5), the anti PrP-P1 antibody R013 (1:1,000) (lane 6), or with R013 preincubated with 25 µg/ml P1 (lane 7). Note the disappearance of the PrP 27-30 immunoreactive bands in lane 7.

## Discussion

We have achieved for the first time the *in situ* immunodetection of PrP<sup>Sc</sup> in scrapie-infected cells. Our results, coupled with those of earlier studies (Stahl et al., 1987), demonstrate that PrP<sup>Sc</sup> and PrP<sup>C</sup> are found in different subcellular compartments. PrP<sup>Sc</sup> accumulates mainly within the cytoplasmic space of cells, whereas PrP<sup>C</sup> is bound primarily to the external surface of the plasma membrane. These observations extend the list of differences which distinguish PrP<sup>Sc</sup> from PrP<sup>C</sup>. Our results also provide novel tools for the investigation of scrapie at the cellular level.

### *In Situ* Immunodetection of PrP<sup>C</sup>

We base the immunodetection of PrP<sup>Sc</sup> on three previously recognized differences between the PrP isoforms: (a) the relative resistance of PrP<sup>Sc</sup> to proteolysis (Oesch et al., 1985); (b) the differential releasability of the PrP isoforms from membranes by PIPLC (Stahl et al., 1990); and (c) the contrasting immunoreactivity patterns of these two proteins (Serban et al., 1990). The immunoreactivity phenomenon was first discovered for PrP immobilized on solid supports: PrP



**Figure 10.** Apparent temperature sensitivity of PrP<sup>Sc</sup> production in HaB cells analyzed by a “colony-assay” for PrP<sup>Sc</sup>. Scrapie-infected and normal HaB and N<sub>2</sub>a cells were seeded on plastic plates in recognizable spot patterns as indicated (a). After 24 h incubation at 37°C, the cells were lysed and blotted onto circular nitrocellulose membranes (each plate yields one filter). One filter was stained with Amido black (a). The two others were treated with proteinase K (50 μg/ml in TBST, 37°C, 18 h), then incubated in PMSF and finally treated with 3 M GdnSCN. The filters were then immunoassayed for PrP by the regular Western blot procedure. The noninfected HaB and N<sub>2</sub>a cells do not react with the PrP 27–30 polyclonal antiserum R073 (1:5,000) in these conditions (b). 13A5 detects only hamster PrP<sup>Sc</sup> and does not recognize the ScN<sub>2</sub>a cells (c).

HaB cells kept at 37°C (d–f) or 34°C (g–i) were incubated with purified hamster prions at an MOI of ~100 ID<sub>50</sub>/cell for 48 h. They were then washed and grown at the above temperatures. After three passages, the cells were seeded on 10-cm plates at low density and incubated until colonies grew to ~1 mm diameter. The cells were then lysed in situ onto nitrocellulose membranes (one membrane per plate) as described in Materials and Methods. The membranes were incubated with proteinase K (e, f, h, and i) followed by a GdnSCN treatment (f and i) to promote selective immunodetection of PrP<sup>Sc</sup> by R073 antibody. Membranes d and g were stained with Amido black to show the colony density. Note that ~100 PrP<sup>Sc</sup>-positive colonies appeared at 34°C (i), whereas only 3 such colonies were apparent at 37.5°C (f).

antibodies raised against SDS-denatured hamster PrP 27–30 recognized equally well both native and denatured PrP<sup>C</sup>, but they did not react with the proteinase K-resistant PrP<sup>Sc</sup>. Extensive denaturation of PrP<sup>Sc</sup> was necessary to promote the binding of antibodies to it (Kascsak et al., 1987; Kitamoto et al., 1987; Serban et al., 1990).

Four lines of evidence argue that the guanidine-dependent PrP signal detected in the three immunoassays described here (the cell ELISA, the immunofluorescent assay, and the colony assay) is mainly PrP<sup>Sc</sup>: (a) it is resistant to proteinase K-catalyzed proteolysis (Table I; Figs. 1 A, 3 A, and 10); (b) it is not removed from the cells by PIPLC (Table I, columns 3 and 7; Figs. 1 and 3 A); (c) its detection depends strictly on protein denaturation (Table I, columns 3 and 7; Figs. 2, 3, and 10); and (d) it occurs only in PrP<sup>Sc</sup>-producing cells or in cells loaded with hamster prion rods. For the cells loaded with rods, the identity of the signal as PrP<sup>Sc</sup> was confirmed by the use of mAbs specific for hamster PrP (Fig. 1, lane 13; Fig. 3 B; Table I, experiment C).

Although the foregoing properties are all hallmarks of PrP<sup>Sc</sup>, we cannot rule out the possibility that some intracellular PrP<sup>C</sup> also participates in the generation of the guanidine-dependent signal.

However, this aberrant PrP<sup>C</sup> would have many of the biochemical properties of PrP<sup>Sc</sup> and appear only in scrapie-infected cells.

The PrP specificity of the signals in our immunoassays was established by the use of (a) rabbit PrP 27–30 antisera R017, R073, and their preimmune sera, (b) mouse mAbs 13A5 and 8C5, and (c) anti-P1 monospecific antiserum R013. Both the ELISA (Table I, experiment B) and immunofluorescence signals (Fig. 5) were abolished by adsorbing the P1 monospecific antiserum with the synthetic oligopeptide P1, whereas the 8C5 signals were adsorbed by the synthetic oligopeptide P5 (Table I, experiment C; Fig. 3 B).

The immunofluorescence assay for PrP<sup>Sc</sup> demonstrates that at least a portion of the PrP<sup>Sc</sup> molecules in scrapie-infected cells is inaccessible to immunological detection before denaturation (Fig. 2). This observation explains why PrP<sup>Sc</sup> has not previously been detected in scrapie-infected cells using immunofluorescence. Furthermore, it indicates that PrP<sup>Sc</sup> in cells assumes a conformation or an aggregation state that precludes its recognition by antibodies raised against the denatured PrP 27–30.

**Table II. Correlation of PrP<sup>Sc</sup> Content with Scrapie Infectivity in ScHaB-4 Cell Subclones**

1	2	3	4
Clones exposed to prions	Cell ELISA (proteinase K + GdnHCl)	Scrapie prion titers for 10 <sup>6</sup> cells	PrP <sup>Sc</sup> immunofluorescence
No.	(A <sub>492</sub> × 10 <sup>3</sup> )	log (ID <sub>50</sub> units ± SE)	
1	310	4.5 ± 0.3	±
2	190	3.3 ± 0.2	+
3	770	ND	+
4	1,090	5.7 ± 0.2	+
5	960	5.0 ± 0.3	+
6	680	5.2 ± 0.3	+
7	110	3.7 ± 0.1	+
8	960	5.3 ± 0.6	+
9	260	4.5 ± 0.3	+
10	430	4.7 ± 0.4	+
11	410	5.3 ± 0.3	+
12	440	5.5 ± 0.2	+
13	610	5.8 ± 0.2	+
14	400	4.9 ± 0.2	+
15	70	<1	-
16	110	<1	-
17	60	1.5 ± 0.9	-
18	80	<1	-
19	10	<1	-
20	30	<1	-
21	50	<1	-
22	20	<1	-
23	50	<1	-
Uninfected HaB cells	30	<1	-

23 subclones of a de novo infected ScHaB-4 cell population were assayed for PrP<sup>Sc</sup> by the cell ELISA assay (column 2) and bioassayed for scrapie infectivity (column 3). Column 4 indicates the presence of a GdnHCl-dependent PrP intracellular signal as detected by immunofluorescence; clone 1 contained both negative and positive cells by this test (±).

### Cytoplasmic Accumulation of PrP<sup>Sc</sup>

The colocalization of PrP<sup>Sc</sup> and WGA ligands in ScN<sub>2</sub>a and ScHaB-4 as well as localization with the mid-Golgi protein MG-160 in ScHaB-4 cells argues that PrP<sup>Sc</sup> accumulates in the periauclear Golgi region (Figs. 6 and 7). However, PrP<sup>Sc</sup> did not localize with MG-160 in the ScHaB-4-C4 subclone (Fig. 8). Although confocal microscopy (White et al., 1987) allowed better subcellular localization, determination of the precise relationship between PrP<sup>Sc</sup> and the Golgi complex or other organelles will require immunoelectron microscopy and subcellular fractionations.

The intracellular accumulation of PrP<sup>Sc</sup> raises interesting possibilities as to the mechanisms of its biosynthesis. Molecular cloning of the Syrian hamster PrP demonstrated that the entire open reading frame is contained within a single exon; this finding argues that the difference between PrP<sup>C</sup> and PrP<sup>Sc</sup> is not due to alternative splicing but instead arises from a posttranslational event (Basler et al., 1986). To date, no recognizable differences in the posttranslational modifications of these proteins have been discerned (Prusiner, 1989). The most convincing evidence for posttranslational processing of PrP<sup>Sc</sup> comes from pulse-chase experiments in scrapie-infected cultured cells. In N<sub>2</sub>a and several other cell types, a fully modified PrP<sup>C</sup> appears on the plasma membrane within 20–30 min of its synthesis, and has a half-life

of ~5 h (Caughey et al., 1989; Borchelt et al., 1990). In contrast, PrP<sup>Sc</sup> acquires its characteristic resistance to proteinase K only several hours after translation (Borchelt et al., 1990). As most PrP<sup>Sc</sup> is then sialylated (Endo et al., 1989) this conversion probably takes place either in the *trans*-Golgi network (Kornfeld and Kornfeld, 1985; Griffiths and Simons, 1986) or in a post-Golgi compartment. At which stage of its biosynthesis does a PrP molecule become committed to be PrP<sup>Sc</sup> is unknown. If pre-PrP<sup>Sc</sup> molecules remain intracellular instead of being exported to the plasma membrane, at a stage when they are not yet proteinase K resistant, then they should already contain the information permitting them to elude the normal sorting mechanisms in the *trans*-Golgi network and hence be already “tagged” and destined to become PrP<sup>Sc</sup> molecules. If this were the case, then the acquisition of proteinase K resistance would occur intracellularly. Alternatively, proteinase K-sensitive PrP<sup>Sc</sup> precursor molecules might first pass through the plasma membrane and acquire their proteinase K resistance either on that membrane or during recycling events (Sniders and Rogers, 1986). In either case the mature proteinase K-resistant PrP would then accumulate either in association with the Golgi apparatus or in post-Golgi, possibly pathologic vesicles.

### Cultured Hamster Cells Permissive for Scrapie Replication

The HaB cells described here constitute the first de novo infectible hamster cell line. That it was isolated from a Syrian hamster is of special interest. First, the incubation time of scrapie in this species is the shortest known. Second, the availability of both mouse and hamster cell lines provides a system in which the species specificity of prions can be studied using molecular genetics (Scott et al., 1989).

Although we found a correlation between the PrP<sup>Sc</sup> content of the subclones and their infectivity (both in the N<sub>2</sub>a and in the HaB cells), some investigators (Race et al., 1988; Caughey et al., 1989) failed to detect any proteinase K-resistant PrP in several infectious subclones of ScN<sub>2</sub>a populations. This discrepancy has been resolved because these investigators have identified PrP<sup>Sc</sup> in their scrapie-infected subclones of N<sub>2</sub>a cells (Race, R., personal communication). The increased sensitivity of the detection methods for PrP<sup>Sc</sup> described here should help unravel future inconsistencies should they arise.

### New Approaches to the Cell Biology of Prion Diseases

The *in situ* PrP<sup>Sc</sup> detection methods described here can be used to search for new scrapie-infectible cell lines and their infected subclones. The ability to localize PrP<sup>Sc</sup> at the subcellular level should permit investigators to follow the entrance of prions into the cell and the subsequent establishment of a chronic scrapie infection as well as the passage of prions from cell to cell. The PrP<sup>Sc</sup> colony assay may provide the basis for the development of tissue culture titration assays for prions (Fig. 10).

Our discovery that PrP<sup>Sc</sup> accumulates within cultured cells should create new approaches to the cell biology of scrapie infection. Learning which inhibitors of glycoprotein processing and transport enhance or diminish PrP<sup>Sc</sup> synthesis and accumulation may give important clues as to the post-translational events which feature in the formation of this

molecule. Furthermore, the results of our studies may aid in determining the subcellular localization of PrP<sup>Sc</sup> in animals and humans with prion diseases. At present, neither the localization nor the physical form of PrP<sup>Sc</sup> in the brain is known except for a small fraction in the extracellular space which is polymerized into amyloid filaments that coalesce to form plaques (DeArmond et al., 1985; Kitamoto et al., 1987; Roberts et al., 1988).

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