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Synthesis and Trafficking of Prion Proteins in Cultured Cells

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Scrapie prions are composed largely, if not entirely, of the scrapie prion protein (PrP^{Sc}) that is encoded by a chromosomal gene. Scrapie-infected mouse neuroblastoma (ScN_2a) and hamster brain (ScHaB) cells synthesize PrP^{Sc} from the normal PrP isoform (PrP^{C}) or a precursor through a posttranslational process. In pulse-chase radiolabeling experiments, we found that presence of brefeldin A (BFA) during both the pulse and the chase periods prevented the synthesis of PrP^{Sc}. Removal of BFA after the chase permitted synthesis of PrP^{Sc} to resume. BFA also blocked the export of nascent PrP^{C} to the cell surface but did not alter the distribution of intracellular deposits of PrP^{Sc}. Under the same conditions, BFA caused the redistribution of the Golgi marker MG160 into the endoplasmic reticulum (ER). Using monensin as an inhibitor of mid-Golgi glycosylation, we determined that PrP traverses the mid-Golgi stack before acquiring protease resistance. About 1 h after the formation of PrP^{Sc}, its N-terminus was removed by a proteolytic process that was inhibited by ammonium chloride, chloroquine, and monensin, arguing that this is a lysosomal event. These results suggest that the ER is not competent for the synthesis of PrP^{Sc} and that the synthesis of PrP^{Sc} occurs during the transit of PrP between the mid-Golgi stack and lysosomes. Presumably, the endocytic pathway features in the synthesis of PrP^{Sc}.

INTRODUCTION

Compelling evidence argues that infectious scrapie prions are composed largely, if not entirely, of the scrapie prion protein (PrP^{Sc})[1] (Prusiner, 1982, 1991; Scott *et al.*, 1989; Hsiao *et al.*, 1990; Prusiner *et al.*, 1990; Weissmann, 1991). The events of PrP^{Sc} biosynthesis are of central importance to understanding the biology of scrapie and similar prion diseases of humans and animals.

PrP^{Sc} accumulates in the brain of humans and animals with prion diseases (Bolton *et al.*, 1982), as well as in cultured cells persistently infected with scrapie (Butler *et al.*, 1988; Borchelt *et al.*, 1990; Caughey *et al.*, 1990; Taraboulos *et al.*, 1990b). PrP^{Sc} is an abnormal isoform of the normal membrane-bound sialoglycoprotein designated the cellular prion protein or PrP^{C} (Oesch *et al.*, 1985). Although the two PrP isoforms differ strikingly in many of their properties, their structural differences are not yet known. Extensive studies failed to reveal chemical differences between the isoforms, pointing to the possibility that these proteins differ in their conformation (Prusiner, 1991; Stahl and Prusiner, 1991). PrP^{Sc} differs from PrP^{C} in its resistance to proteolysis and its insolubility in detergents (Meyer *et al.*, 1986). Limited digestion of PrP^{Sc} removes the N-terminal 67 amino acids to produce PrP 27–30 (Prusiner *et al.*, 1984; Oesch *et al.*, 1985).

Cultured cells persistently infected with scrapie offer a convenient system to study the biogenesis of the prion protein isoforms. Studies involving mouse neuroblastoma (N_2a) cells (Butler *et al.*, 1988; Caughey *et al.*, 1990) and hamster brain (HaB) cells (Taraboulos *et al.*, 1990b) have shown the following. 1) PrP^{C} is transported to the

[1] Abbreviations used: BFA, brefeldin A; GdnSCN, guanidine thiocyanate; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PIPLC, phosphatidylinositol-specific phospholipase C; PMSF, phenylmethylsulfonyl fluoride; PrP^{C}, cellular isoform of the prion protein; PrP^{Sc}, scrapie isoform of the prion protein; ScHaB, scrapie-infected hamster brain; ScN_2a, scrapie-infected mouse neuroblastoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; $t_{1/2}$, half-life time; Tris, tris(hydroxymethyl)aminomethane.

cell surface within 1 h of its synthesis (Caughey *et al.*, 1989; Borchelt *et al.*, 1990). 2) The proteinase K resistance of PrP^{Sc} and its insolubility in detergents are acquired posttranslationally (Borchelt *et al.*, 1990; Taraboulos *et al.*, 1990a; Caughey *et al.*, 1991) with a half-life time ($t_{1/2}$) ~ 1 h in scrapie-infected Syrian hamster (ScHaB) cells and $t_{1/2}$ ~ 3 h in scrapie-infected mouse neuroblastoma (ScN_2a) cells (Borchelt *et al.*, 1992). 3) in ScN_2a cells, PrP^{Sc} is derived from a plasma membrane bound precursor (Caughey and Raymond, 1991; Borchelt *et al.*, 1992). 4) Although PrP^{C} is attached to the plasma membrane of cells by a C-terminal phosphoinositol glycolipid anchor (Stahl *et al.*, 1987), PrP^{Sc} accumulates intracellularly (Taraboulos *et al.*, 1990b), partly in secondary lysosomes, some of which contain acid phosphatase (McKinley *et al.*, 1991b). Whether PrP^{Sc} is similarly anchored to lipid bilayers in the cell is unknown. 5) Protease-resistant PrP can be synthesized in the absence of Asn-linked glycosylation, and therefore structural differences unrelated to these carbohydrates must exist between these isoforms (Taraboulos *et al.*, 1990a).

To delineate the cellular pathways utilized during the biosynthesis of protease-resistant PrP^{Sc} and to gain information about the structural events involved in the conversion of PrP^{C} to PrP^{Sc}, we used brefeldin A (BFA) to prevent exit of proteins from the endoplasmic reticulum (ER)-Golgi (Misumi *et al.*, 1986; Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). We found that BFA blocks reversibly the synthesis of PrP^{Sc}. These observations demonstrate that the ER-Golgi is insufficient for synthesis of PrP^{Sc}. Deciphering the reasons for the inability of the ER-Golgi to catalyze this process may help elucidate the structural changes in PrP that occur during the formation of PrP^{Sc}. Furthermore, intracellular PrP^{Sc} deposits previously localized by immunocytochemistry to secondary lysosomes do not redistribute into the ER-Golgi during exposure to BFA (McKinley *et al.*, 1991b). It is possible that the ER-Golgi of BFA-treated cells, although not inherently unable to synthesize PrP^{Sc}, lacks an essential scrapie-specific molecule such as PrP^{Sc} itself. Results from transgenic mouse studies contend that PrP^{C}-PrP^{Sc} complexes feature in the formation of nascent PrP^{Sc} (Prusiner *et al.*, 1990). Indeed, PrP^{Sc} seems to be acting as a "template" for generating more of itself from the cellular PrP isoform (Prusiner, 1991). BFA is the first compound found to inhibit reversibly the synthesis of PrP^{Sc}.

Using monensin to inhibit mid-Golgi glycosylation, we determined that PrP^{Sc} molecules acquired protease resistance after traversing this Golgi cisterna. Neither monensin nor lysosomotropic amines inhibited the formation of PrP^{Sc}, indicating that vacuolar acidic pH is not essential for PrP^{Sc} synthesis. About 1 h after acquiring its protease-resistant core, PrP^{Sc} undergoes an N-terminal trimming that can be inhibited by lysosomotropic amines and monensin, indicating that this limited proteolysis occurs in an acidic degradative compartment, probably the endocytic system or lysosomes. Lysosomal processing of PrP^{Sc} is consistent with the finding that this protein accumulates at least in part within secondary lysosomes in both ScN_2a and ScHaB cells (McKinley *et al.*, 1991b).

MATERIALS AND METHODS

Materials

Reagents for cell cultures were purchased from UCSF's Cell Culture Facility, except the methionine-free Dulbecco's modified Eagle's medium (DMEM) and the reduced serum medium Opti-MEM, which were from GIBCO (Grand Island, NY). Tunicamycin and fluoresceinated secondary antibodies were from Boehringer Mannheim (Indianapolis, IN). Monensin and forskolin were from Calbiochem (La Jolla, CA). BFA was purchased from Epicentre Technologies (Madison, WI). Guanidine thiocyanate (GdnSCN) was from Fluka Chemical (Buchs, Switzerland). ^{35}S L-methionine and the fluor "Amplify" were from Amersham (Arlington Heights, IL). Phosphatidylinositol-specific phospholipase C (PIPLC) was prepared from *Bacillus Thurigensis* as previously described (Low *et al.*, 1988). Endoglycosidase H was from New England Nuclear (Boston, MA). The Protoblot system for Western immunodetection was purchased from Promega (Madison, WI). Protein A-sepharose was from Pharmacia (Piscataway, NJ). All other chemicals were from Sigma (St. Louis, MO).

Cells

N_2a cells were obtained originally from the American Tissue Culture Collection (Rockville, MD). ScN_2a cells are the persistently infected clone described by Butler *et al.*, 1988. HaB cells were derived from the culture of a Syrian hamster brain as described (Taraboulos *et al.*, 1990b). ScHaB cells are the persistently infected clone ScHaB-4-C4 described earlier (Taraboulos *et al.*, 1990b). ScHaB cells were passaged every 10 days at a 1:5 dilution. Their PrP^{Sc} content varies with time and some batches tend to cease producing PrP^{Sc} altogether (Taraboulos *et al.*, 1990b). All the cells were grown and maintained at 37.5°C in DME-H16 plus 10% fetal calf serum.

Antibodies

R073 is an antiserum raised in a rabbit against sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-purified Syrian hamster PrP 27–30 (Serban *et al.*, 1990). R002 and R013 are rabbit antisera directed against the PrP peptides P2 and P1, respectively (Barry *et al.*, 1988). These antibodies react with both hamster and mouse PrP, and their specificity in immunoprecipitation and immunocytochemistry has been described elsewhere (Borchelt *et al.*, 1990; Serban *et al.*, 1990; Taraboulos *et al.*, 1990a,b). R002 does not react with PrP27-30 because its epitope is missing from this protease-resistant core. The monoclonal antibody 3F4 (Kascsak *et al.*, 1987) recognizes Syrian hamster PrP. Rabbit MG160 antiserum raised against the rat brain mid-Golgi protein MG160 was a generous gift from Dr. Nicholas Gonatas (University of Pennsylvania, Philadelphia, PA) (Gonatas *et al.*, 1989).

Radioactive Labeling and Inhibitors

Confluent cells growing on 5-cm plates were rinsed three times with phosphate-buffered saline (PBS) and incubated for 30 min with methionine-free DME-H16 medium. The medium was then replaced with fresh methionine-free DMEM supplemented with 0.5 mCi/ml of ^{35}S L-methionine (Amersham). At the end of the labeling period, the cells were rinsed twice with DMEM and then either lysed and processed for immunoprecipitation, or first chased in DMEM plus 10% fetal calf serum or in Opti-MEM, as indicated, for the appropriate

length of time. Inhibitors were added either to the pulse or the chase periods, or both, as necessary. Inhibitors used in the pulse period were also added to the methionine starving period.

BFA was stored as a 5-mg/ml stock solution in ethanol (at −20°C) and was used at 5 μg/ml except where otherwise indicated. Monensin was stored as a 10-mg/ml stock in ethanol (at −20°C) and used at 30 μM. Chloroquine and ammonium chloride were prepared fresh for each experiment and used at 10 and 30 μM, respectively. Tunicamycin was prepared in dimethyl sulfoxide at 10 mg/ml just before use. It was used at 5 μg/ml for HaB cells and 30 μg/ml in N_2a cells (Taraboulos *et al.*, 1990a).

Cell Lysis and Immunoprecipitation

The cells were processed for immunoprecipitation as described elsewhere (Taraboulos *et al.*, 1990a) with slight modifications. For immunoprecipitation of total PrP, the cells were lysed in 1 ml ice-cold lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.8), the insoluble material was removed by a low-speed centrifugation, and the lysates were digested with proteinase K (50 μg/ml, 37°C, 1 h) (if needed) before GdnSCN denaturation and methanol precipitation. The methanol pellets were resuspended for immunoprecipitation in 1 ml TNS (100 mM NaCl, 1% sodium sarcosinate [Sarkosyl], 10 mM Tris-HCl, pH 7.8) supplemented with 0.1 mM phenylemethylsulfonyl fluoride (PMSF). One microliter of antiserum and 100 μl of 1% bovine serum albumin were then added for a 12- to 18-h incubation. IgG was insolubilized with protein A-sepharose. For the purification of PrP from cell or membrane supernatants, Sarkosyl and PMSF were added to a final concentration of 1% and 0.1 mM, respectively, before immunoprecipitation as described above.

Purification of Insoluble PrP

Cells were lysed in 1 ml lysis buffer supplemented with 0.1 mM PMSF. After removal of nuclei and debris in a low-speed spin, the lysate was subjected to a high-speed centrifugation (40 000 rpm, 1 h, 4°C in a TL-100 rotor, Beckman, Fullerton, CA). The pellet was rinsed once in TNS and then solubilized in 3 M GdnSCN, 1% Sarkosyl, 10 mM Tris, pH 7.8, 0.1 mM PMSF (37°C, 12–18 h). The GdnSCN was removed by precipitation with ethanol, and the proteins were resuspended in TNS for immunopurification as described above.

Endoglycosidase H Digestion

Immunopurified PrP was removed from the protein A-sepharose by boiling for 3 min in 50 μl 0.1 M Na citrate (pH 5.5), 0.1% SDS. The supernatants were divided into two aliquots. Endoglycosidase H was added to one aliquot to a final concentration of 1 U/ml, and both aliquots were incubated at 37°C for 16 h. After digestion, samples were dryed and were processed for PAGE.

Digestion of Membranes with PIPLC

For release of plasma membrane proteins on intact cells in serum-free medium, PIPLC (10 μU/ml) was added to the cell medium for the period of the radiolabeling. For digestion of total membranes with PIPLC, labeled cells were scraped into a pyrex tube and disrupted by sonication in a bath sonicator for 30 s. Nuclei and cell debris were pelleted in a low-speed centrifugation. To strip the membranes of peripheral proteins, sodium carbonate was added to the supernatant to a final concentration of 100 mM. After a 5-min incubation on ice, the membranes were pelleted for 45 min at 100 000 × *g* in a TL-100 rotor (Beckman). The pellet was rinsed twice with 100 mM Tris-HCl, pH 6.9, resuspended by sonication in PBS supplemented with 100 mM Tris-HCl, pH 6.9, and digested with PIPLC (10 μU/ml) for 12 h at room temperature, and the membranes were again sedimented by ultracentrifugation. Immunoprecipitation of PrP released into the supernatant was accomplished as described above.

Immunofluorescence

Indirect immunofluorescence assays for PrP and the Golgi protein MG160 (Gonatas *et al.*, 1989) were performed as described (Taraboulos *et al.*, 1990b). Cells grown on eight-well glass slides (Nunc, Roskilde, Denmark) were fixed for 30 min with 3.7% formaldehyde in PBS (1.5% when anti-peptide antibodies were used), permeabilized for 1 min with 0.4% Triton X100 in PBS, treated for 5 min with 3 M GdnSCN to denature PrP^{Sc}, blocked with 5% milk in PBS for 30 min, and then incubated sequentially with the primary antibody (R073, R013, and R002, 1:1000; anti-MG160, 1:500) and with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Boehringer Mannheim). The antibodies were diluted in 5% nonfat milk in PBS.

SDS-PAGE and Western Immunoblotting

SDS-PAGE was performed as described by Laemmli (1970). We used 12% acrylamide gels except for the experiments described in Figure 1, C and D where 15% gels were used. For Western blot analysis, the proteins were electrotransferred (Towbin *et al.*, 1979) to nitrocellulose paper. The membrane was blocked with 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 (Promega, Madison, WI).

RESULTS

Nomenclature

Although PrP^{Sc} becomes resistant to proteases only several hours after the synthesis of the PrP polypeptide chain, we do not know at what point its protease-sensitive precursor diverges from the PrP^{C} pool and becomes committed to the generation of PrP^{Sc}. We envisage at least two possible scenarios: PrP^{C} is the direct precursor of PrP^{Sc} and the transformation of PrP^{C} into PrP^{Sc} occurs in a single step or PrP molecules destined to become PrP^{Sc} separate from PrP^{C} early in the biosynthetic pathway. Although indistinguishable from PrP^{C} by their resistance to proteases or their solubility in detergents (Borchelt *et al.*, 1990; Taraboulos *et al.*, 1990a; Caughey and Raymond, 1991), these PrP precursor molecules could conceivably be subject to different sorting and processing. We refer to the protease-sensitive precursor of PrP^{Sc} as the "PrP^{Sc} precursor." In some cases, the generic name "PrP" is used to designate all PrP chains, including the PrP^{Sc} precursor. Also, we refer to the formation of protease-resistant PrP^{Sc} simply as "PrP^{Sc} synthesis."

BFA Reversibly Inhibits PrP^{Sc} Synthesis

Treatment of cells with the fungal antibiotic BFA results in the rapid but reversible disappearance of the *cis*-, medial-, and *trans*-Golgi cisternae as they fuse with the ER (Fujiwara *et al.*, 1988; Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989, 1990; Chege and Pfeffer, 1990). As a result, protein export from the ER is blocked (Misumi *et al.*, 1986; Oda *et al.*, 1987). In addition, BFA

induces mixing of the trans Golgi network (TGN) with the early endosomal system (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991). We studied the effect of BFA on the synthesis of the PrP isoforms. ScHaB cells (Figure 1A, lanes 1–9) and ScN_2a cells (Figure 1A, lanes 10–13) were radiolabeled for 1 or 2 h, respectively, in the presence or absence of BFA (5 μg/ml) as indicated and then either immediately analyzed by PrP immunoprecipitation or first chased for 6 (ScHaB) or 12 (ScN_2a) additional h in unlabeled medium with or without BFA. PrP synthesized in the presence of BFA appeared as a broad band in the region of 30 kDa (lane 2, no proteolysis). Cells radiolabeled in the presence of BFA, but chased in its absence, produced a 19- to 27-kDa protease-resistant PrP^{Sc} core (Figure 1A, lanes 7 and 12). In the case of the ScHaB cells, this core could also be immunoprecipitated with the anti-PrP monoclonal antibody 3F4 (Kascsak *et al.*, 1987), confirming its identity. The reduced M_r of the 19- to 27-kDa core as compared with PrP^{Sc} synthesized in unperturbed cells (lane 5) is probably due to the aberrant glycosylation of the protein in the presence of BFA. In contrast, when BFA was present also during the chase period, no protease-resistant PrP species could be detected in the cells (lanes 8 and 13). This complete inhibition did not appear to be due primarily to a direct effect of the drug on the process of protease-resistance acquisition, because cells radiolabeled without BFA and chased in its presence did produce PrP^{Sc}, albeit at a reduced level (lanes 6 and 11).

PrP^C and the PrP^{Sc} Precursor are Stable in Cells Treated with BFA

BFA inhibition of PrP^{Sc} synthesis could be explained if PrP was labile in the ER of BFA-treated cells; in fact, an ER degradation pathway has been described (Lippincott-Schwartz *et al.*, 1988; Wikstrom and Lodish, 1991). To address this possibility, we examined the stability of PrP^C in BFA-treated cells. ScN_2a cells were radiolabeled for 1 h and chased for the indicated periods of time in the presence of BFA and PrP^C analyzed (without proteolysis) (Figure 1C). We found that the stability of PrP^C was increased, rather than decreased, when the cells were continuously exposed to BFA. The $t_{1/2}$ for PrP^C degradation in untreated cells is ~3–6 h (Caughey *et al.*, 1989; Borchelt *et al.*, 1990). However, because the PrP^{Sc} precursor may not be identical with PrP^C, we verified directly the stability of this precursor. ScHaB cells were radiolabeled for 1 h in the presence of BFA, chased for 12 h in the presence of the drug, and then chased for an additional 8-h period without BFA. Under these conditions, the cells still synthesized some protease-resistant PrP^{Sc} (Figure 1A, lane 9), indicating that the PrP^{Sc} precursor was still present in the cell after the first 8-h chase with BFA. Thus, depletion of PrP in cells chased with BFA is not the mechanism preventing the synthesis of protease-resistant PrP^{Sc}.

PrP Becomes Resistant to Endoglycosidase H in the Presence of BFA and Thus is Exposed to Golgi Enzymes

In cells treated with BFA, newly synthesized proteins retained in the ER acquire endoglycosidase H-resistant oligosaccharides, witnessing their exposure to medial Golgi processing enzymes that have redistributed into the ER (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989). Endoglycosidase H cleaves "high mannose" but not "complex" type oligosaccharides (Tarentino and Maley, 1974; Kornfeld and Kornfeld, 1985). We confirmed that this is also true for PrP in ScN_2a cells. ScN_2a cells were pulse radiolabeled for 1 h and chased for increasing periods of time in the presence of BFA (5 μg/ml) (Figure 1B). After immunopurification, PrP was digested with endoglycosidase H and its M_r compared with that of undigested PrP extracted from the same cells. Resistance to endoglycosidase H was complete 4 h after the end of the pulse period (Figure 1B). Thus, exposure of PrP to redistributed Golgi enzymes in the ER is not sufficient for the formation of PrP^{Sc}.

BFA does not Redistribute PrP^{Sc} into the ER but Blocks PrP^C Export to the Plasma Membrane

ScHaB cells grown on glass slides were treated with 5 μg/ml BFA or the diluent alone, and the distributions of the mid-Golgi protein MG160 (Gonatas *et al.*, 1989) and of PrP^{Sc} were examined by immunofluorescence (Figure 2A). In BFA-treated cells, MG160, but not PrP^{Sc}, redistributed into a reticular configuration characteristic of the ER. Thus, the mixed ER-Golgi seems to be devoid of PrP^{Sc}. This finding complements our ultrastructural studies that showed the accumulation of PrP^{Sc} in cytoplasmic vesicles, some of which were identified as secondary lysosomes (McKinley *et al.*, 1991b). The apparent absence of PrP^{Sc} in the ER-Golgi is important in view of transgenic mouse studies that argue that PrP^{Sc} synthesis involves the formation of PrP^C-PrP^{Sc} complexes (Prusiner *et al.*, 1990) and thus may require existing PrP^{Sc} molecules.

To confirm that BFA indeed prevented export of PrP to the plasma membrane, we examined its accessibility to the enzyme PIPLC added to the cell medium (Figure 2B). ScHaB cells were radiolabeled for 3 h in the presence (lanes 3 and 4) or the absence (lanes 1 and 2) of BFA. In lanes 2 and 4, PIPLC (10 μU/ml) was added to the labeling medium. PIPLC could release PrP^C from untreated cells, as expected (Stahl *et al.*, 1987), but not from cells labeled in the presence of BFA (lane 4). That BFA did not inhibit PrP glypiation was demonstrated by studies showing release of PrP^C from microsomes prepared from cells incubated with BFA after exposure to PIPLC (lane 8).

PIPLC released more PrP from microsomes derived from untreated cells (lane 6) than from vesicles from BFA-treated cells (lane 8). This may be due to the dif-

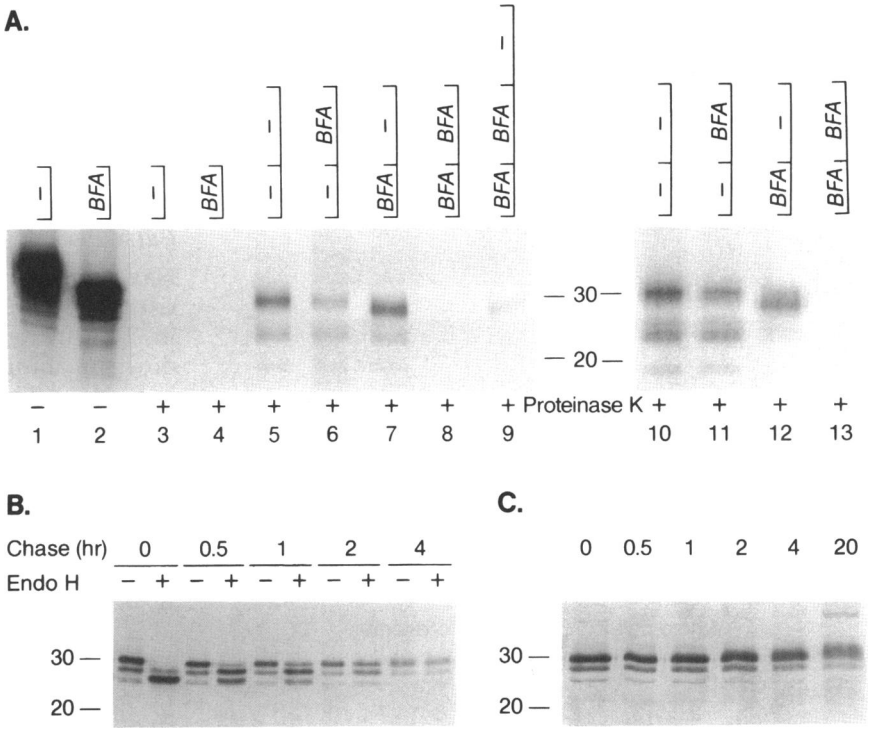

Figure 1. Brefeldin A (BFA) reversibly inhibits synthesis of PrP^{Sc}. (A) ScHaB (lanes 1–9) and ScN_2a (lanes 10–13) cells were radiolabeled for 1 or 2 h, respectively, and then either lysed for PrP analysis (lanes 1–4) or first chased for an additional 6 h (ScHaB) or 12 h (ScN_2a). BFA (5 μg/ml) was added to the pulse or the chase period, or both, as indicated. In lanes 1 and 2, proteinase K digestion was omitted to permit the analysis of both PrP isoforms; all other samples were subjected to proteolysis catalyzed by proteinase K (50 μg/ml, 37°C, 1 h) before immunoprecipitation with R073 antiserum. Presence of BFA in both the pulse and the chase media resulted in the complete inhibition of protease-resistant PrP^{Sc} (lanes 8 and 13). In lane 9 of A, the cells were first chased for 12 h in the presence of BFA and then the chase was continued for 8 additional h without the inhibitor. (B and C) N_2a cells were radiolabeled for 1 h and then chased for the indicated periods of time, in the presence of 5 μg/ml BFA. PrP was then analyzed without proteolysis. In B, immunopurified PrP was digested with endoglycosidase H before electrophoresis.

ferent location of PrP in the two cases. Although most PrP^{C} is expected to be localized primarily inside vesicles derived from BFA-treated cells, the protein ought to be found mainly on the outside of vesicles from untreated cells and thus more accessible to PIPLC cleavage.

PrP^{Sc} Synthesis and PrP^{C} Export to the Plasma Membrane are Inhibited at the same BFA Concentration

To determine whether the inhibition of PrP^{Sc} synthesis by BFA is related to the effects of the drug on the secretory pathway, we studied the dependence of these processes on the concentration of BFA added to the cell medium (Figure 3). ScN_2a cells were pulse-radiolabeled for 2 h and then chased for 7 h in Opti-MEM, in the presence of increasing concentrations of BFA (from 10 ng/ml, lane 2, to 2.5 μg/ml, lane 9; lane 1, no BFA). The cells were then analyzed for radiolabeled protease-resistant PrP^{Sc} (Figure 3A). PrP^{Sc} synthesis was inhibited by a BFA concentration of 160 ng (lane 6). The residual band appearing underneath the 19-kDa PrP^{Sc} band even at high BFA concentrations did not appear in other experiments (Figure 1A) and is probably unrelated to PrP. To measure the amount of PrP^{C} exported to the plasma membrane under these conditions, cells in a parallel experiment were treated with PIPLC during the chase period, and the chase medium was analyzed for released PrP^{C} (Figure 3B). The radioactivity in the PrP bands in both gels is plotted in Figure 3C. Both PrP^{Sc} synthesis and PrP^{C} export to the plasma membrane were inhibited at the same BFA concentration. These results support the contention that the inhibition of PrP^{Sc} synthesis by BFA is not due to a direct interaction between the drug and proteins catalyzing the synthesis of PrP^{Sc} but rather to the effects of BFA on the secretory pathway. The simplest interpretation of our results is that the mixed ER-Golgi system is unable to synthesize PrP^{Sc} and that the PrP^{Sc} precursor, possibly PrP^{C}, has to exit from this organelle before PrP^{Sc} can be synthesized. Interestingly, the ^{35}S incorporation in the 30-KDa band of PrP^{Sc} increased at subinhibitory BFA levels. We surmise that this may be due to the altered glycosylation pattern of PrP at these BFA concentrations. Reducing PrP glycosylation increases the synthesis of PrP^{Sc} (Taraboulos *et al.*, 1990a).

Blocking Translation During the Chase Period Inhibits PrP^{Sc} Synthesis

Because many of the cellular inhibitors used in our investigation inhibit protein synthesis to some extent, we sought to determine whether translation inhibitors applied to the cells during the chase period modify the formation of PrP^{Sc}. ScN_2a cells were radiolabeled for 2 h and then chased for 8 h in the presence (Figure 4, lane 2) or absence (lane 1) of 20 μg/ml cycloheximide. ^{35}S-methionine incorporation into trichloroacetic acid precipitable material was decreased by ~97% in these conditions. The formation of protease-resistant PrP^{Sc} was strongly inhibited by the cycloheximide (lane 2). Identical results were obtained when puromycin was

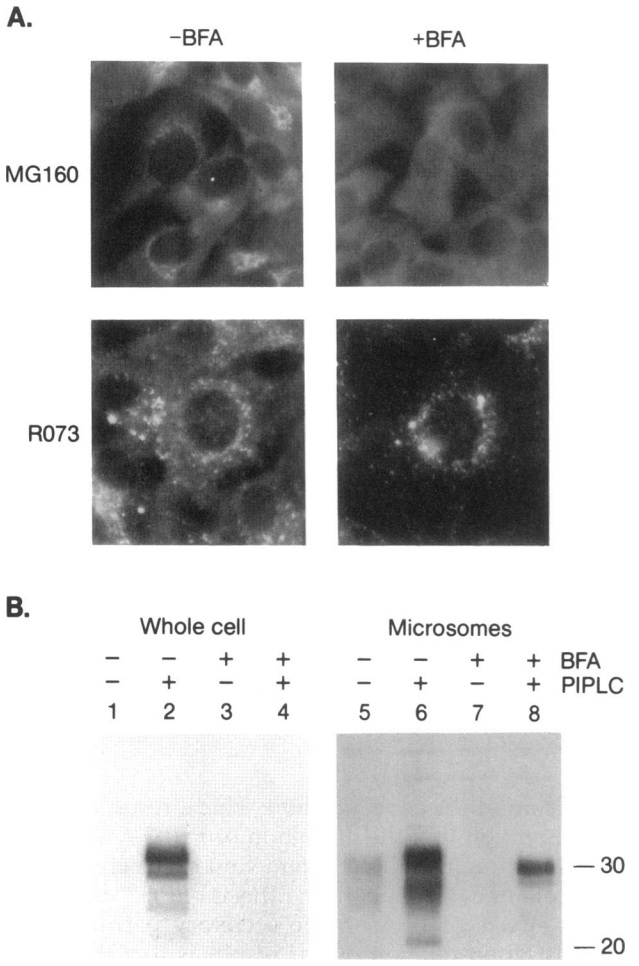

Figure 2. BFA blocks export of newly synthesized PrP^C to the plasma membrane but does not redistribute PrP^{Sc} into the ER. (A) ScHaB cells grown on glass slides were treated with BFA (5 μg/ml) for 2 h or left untreated as indicated and were then labeled by immunofluorescence for the mid-Golgi protein MG160 (MG160) or PrP^{Sc} (R073). After BFA treatment, MG160 lost its perinuclear distribution to assume a reticular pattern expanding throughout the cell. In contrast, PrP^{Sc} retained its speckled distribution. (B) ScHaB cells were radiolabeled for 3 h. In lanes 3, 4, 7, and 8, BFA (5 μg/ml) was added to the labeling medium. In lanes 1–4, the cell supernatant was analyzed for the presence of PrP. In lanes 2 and 4, PIPLC (10 μU/ml) was included in the labeling medium. BFA completely prevented the release of PrP by PIPLC (compare lane 4 to lane 2). In lanes 5 to 8, microsomes prepared from the cells at the end of the radiolabeling period were examined for PIPLC-releasable PrP^C. Addition of BFA in the pulse period did not prevent the PIPLC catalyzed release of PrP^C from microsomes (lane 8) and hence did not inhibit PrP glypiation.

used to block translation. One can envisage two possible reasons for this phenomenon: one or more labile proteinaceous cofactors could be necessary for PrP^{Sc} conversion or the general degradation of cellular function brought about by the long exposure to cycloheximide or puromycin inhibits PrP^{Sc} synthesis. This observation may explain the partial inhibition in PrP^{Sc} synthesis observed when BFA was added to the chase period (Figure 1A, lanes 5 and 12), because this inhibitor, although leaving protein translation almost unaffected in ScN_2a, effectively blocks the supply of many newly synthesized proteins to cellular compartments beyond the ER-Golgi where PrP^{Sc} synthesis seems to occur.

PrP^{Sc} Precursor Traverses the Mid-Golgi Within the Pulse Period

Having established that the acquisition of protease resistance by PrP^{Sc} does not occur in the mixed ER-Golgi in BFA-treated cells, we examined compartments downstream in the secretory pathway. PrP^{Sc} contains complex *N*-linked carbohydrates (Endo *et al.*, 1989; Haraguchi *et al.*, 1989) and therefore has to traverse the Golgi apparatus during its biosynthesis. In uninfected N_2a and HaB cells, most PrP^C is fully glycosylated within 30 min of its synthesis (Caughey *et al.*, 1989; Borchelt

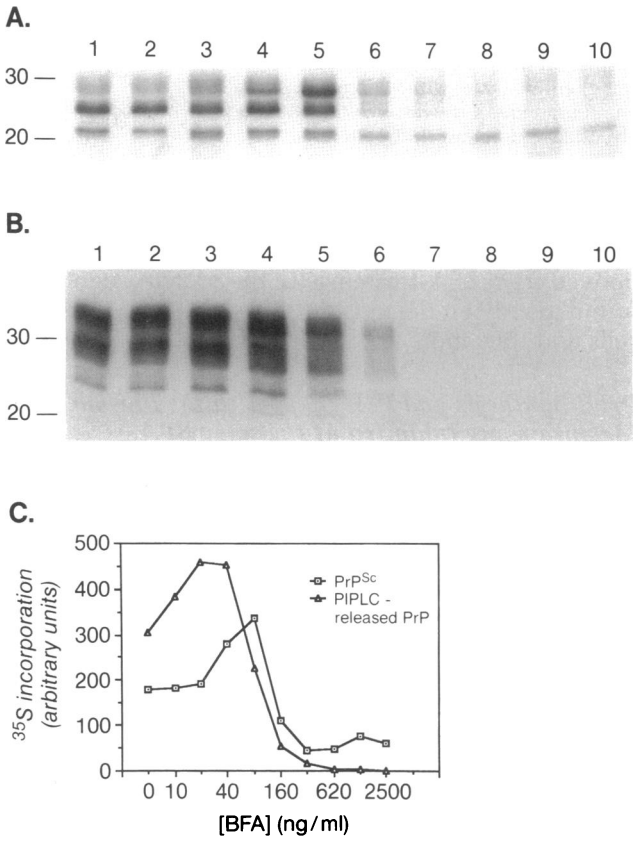

Figure 3. Dependence of PrP^{Sc} synthesis and PrP export to the cell surface on BFA concentration. ScN_2a cells were radiolabeled for 2 h and chased for 7 h in Opti-MEM in the presence of increasing concentrations of BFA (from 10 ng/ml in lane 2 to 2.5 μg/ml in lane 10. Lane 1, no BFA) (A) The cells were lysed and analyzed for PrP^{Sc} synthesis after proteinase K digestion. (B) In other plates, PIPLC (10 μU/ml) was added to the chase medium, which was analyzed for released PrP. (C) Plot of the radioactivity in the PrP bands in gels A and B. The radioactivity was measured using the Phosphorlmager technology (Molecular Dynamics).

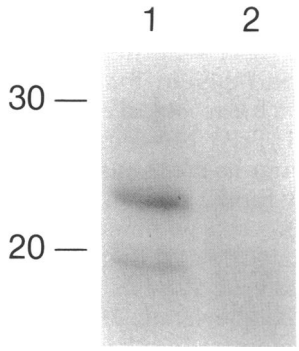

Figure 4. Translation inhibitors in the chase period interfere with PrP^{Sc} maturation. ScN_2a cells were radiolabeled for 2 h and then chased for 8 h in the presence (lane 2) or absence (lane 1) of 20 μg/ml cycloheximide. The cells were lysed and analyzed for PrP^{Sc} synthesis after proteinase K digestion.

et al., 1992). PrP^C glycosylation is thus completed long before PrP^{Sc} appears in scrapie-infected cells. On the other hand, because the PrP^{Sc} precursor may differ from PrP^C, we cannot be certain of its kinetics of glycosylation, and thus we cannot conclude that Golgi glycosylation precedes PrP^{Sc} synthesis. To address this issue, we used monensin as an inhibitor of mid-Golgi glycosylation. This Na/H ionophore releases pH gradients throughout the cell and disrupts the functions of the mid-Golgi cisterna (Tartakoff and Vassalli, 1978). In some, but not all, cases, protein secretion is also disrupted, and transiting proteins accumulate in the mid-Golgi (Griffiths *et al.*, 1983).

To determine whether monensin blocks export of PrP to the plasma membrane (Figure 5A), we used PIPLC, PrP^C synthesized in ScHaB cells treated with up to 30 μM monensin could be released into the medium by PIPLC, indicating that this ionophore did not prevent the passage of PrP^C through the mid-Golgi stack. However, the reduced M_r witnessed its aberrant glycosylation when synthesized in the presence of monensin.

To assess the effect of monensin on PrP^{Sc} synthesis, pulse-chase experiments were used (Figure 5B). ScHaB cells were labeled for 1 h and chased for 4 h, as indicated. The presence of monensin (30 μg/ml) in the medium during the pulse-chase resulted in a protease-resistant PrP polypeptide with a reduced M_r of 19–26 kDa. In contrast, when the cells were pulse-radiolabeled in the absence of monensin but chased in its presence, PrP^{Sc} displayed a normal heterogeneous M_r of 19–30 kDa. We conclude that mid-Golgi glycosylation of PrP occurred during the pulse period, hence, before PrP^{Sc} synthesis. This conclusion applies only to the glycoforms of PrP^{Sc} and may not reflect the 19-kDa unglycosylated PrP^{Sc} (Taraboulos *et al.*, 1990a) that cannot be tracked in this way. Furthermore, that monensin in the chase period did not significantly inhibit the synthesis of PrP^{Sc} suggests that vacuolar acidic pH is not essential for this process to occur. A similar result was obtained with lysosomotropic amines as described below.

These results also show that the transport of PrP^{Sc} precursor through the secretory pathway occurs within the same time frame as that of PrP^C, supporting the view that PrP^C is the precursor of PrP^{Sc}.

PrP^{Sc} Loses its N-Terminus Shortly After its Synthesis

PrP immunocytochemistry has revealed that in both ScHaB and ScN_2a cells, PrP^{Sc} accumulates, at least in part, in acid phosphatase-positive vesicles where it is presumably exposed to acid hydrolases within secondary lysosomes (McKinley *et al.*, 1991b). Because the 67 N-terminal residues of PrP^{Sc} are known to be sensitive to a variety of proteases (Basler *et al.*, 1986; Prusiner *et al.*, 1984), we sought to determine their fate when the radiolabeled cells were chased for longer periods. ScHaB cells were radiolabeled for 1 h and then chased for 2–8 h as indicated (Figure 6A). To avoid the use of proteases in the purification of PrP^{Sc}, we took advantage of PrP^{Sc} insolubility in detergents (Meyer *et al.*, 1986)

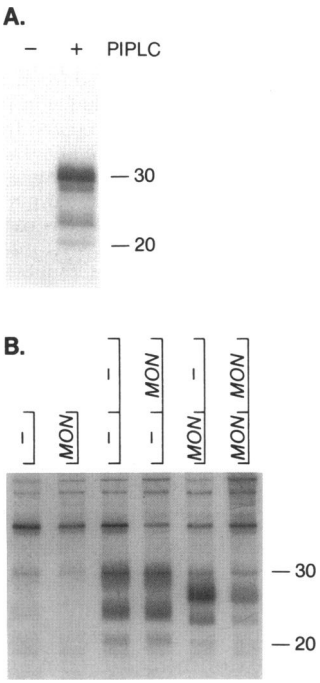

Figure 5. Synthesis of protease-resistant PrP in the presence of monensin. (A) ScHaB cells were pulse-radiolabeled for 3 h in the presence of monensin (30 μM), and the cell supernatant was analyzed for released PrP. In lane 2, PIPLC (10 μU/ml) was included in the labeling medium. Monensin did not prevent export of PrP to the cell surface. (B) ScHaB cells were pulsed for 1 h and chased for 4 h in the presence of monensin (30 μM) as indicated. Monensin did not prevent the formation of protease-resistant PrP (the three bands of M_r between 19 and 31 kDa). PrP synthesized in the presence of the drug exhibited a reduced M_r probably due to aberrant glycosylation.

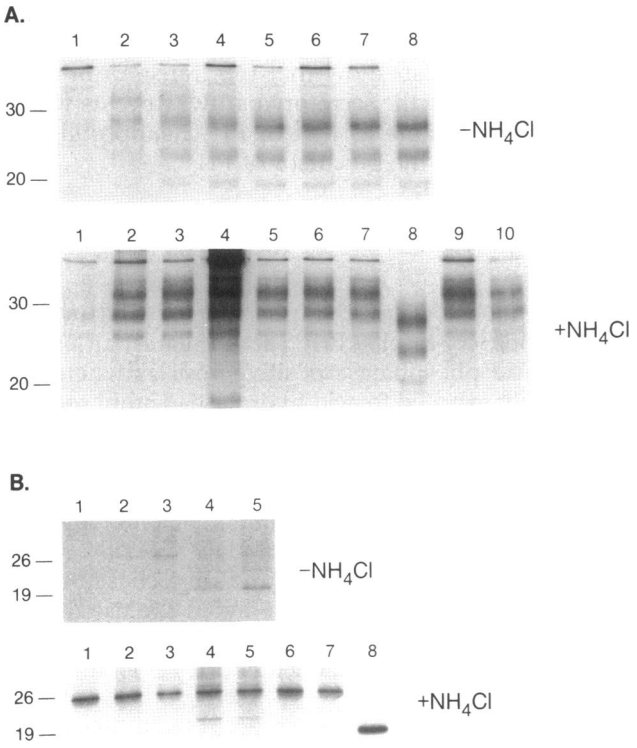

Figure 6. Lysosomal inhibitors prevent the N-terminal degradation of PrP^{Sc} but not its synthesis. ScHaB (A) and ScN_2a (B) cells were pulse-radiolabeled for 1 h and then chased for increasing periods of time. In B, tunicamycin (30 μM) was included in the pulse medium. The cells were then lysed. PrP^{Sc} was separated from PrP^{C} by sedimentation (100 000 × *g*, 1 h, 4°C) before immunoprecipitation and analysis, except in lane 8 where cell lysates were subjected to proteolysis (proteinase K, 50 μg/ml, 37°C, 1 h) but not to sedimentation, before PrP immunoprecipitation. (A) The cells were chased for 0, 1, 2, 3, 4, or 5 h (lanes 1–6) or 6 h (lanes 7–10). In the lower panel (+NH_4Cl), NH_4Cl (30 μM, lanes 1–8), chloroquine (100 μM, lane 9), or monensin (30 μM, lane 10) were included during both pulse and chase. (B) The cells were chased for 0, 1, 2, 3, 4, or 5 h (lanes 1–6) or 6 h (lanes 7 and 8). In the lower panel, + NH_4Cl (30 μM) was present during both pulse and chase periods.

to separate it from PrP^{C}. After removal of large insoluble material in a low-speed spin, the cell lysate was subjected to a 100 000 × *g* centrifugation for 1 h, and PrP^{Sc} was immunoprecipitated from the pellet and analyzed by SDS-PAGE.

Immediately after the pulse, three weak insoluble PrP^{Sc} bands could be detected in the 26- to 35-kDa region (lane 1). On extending the chase, the M_r of this triplet was reduced to 27–30, 24, and 19 kDa (lanes 2–7), coinciding with that obtained after limited digestion with proteinase K (lane 8). The reduction in M_r was accompanied by the loss of the N-terminus of PrP because the molecule could no longer be precipitated with R002, an antibody directed against the amino terminal peptide P2. Addition of NH_4Cl (30 μM) (Figure 6A, +NH_4Cl, lanes 1–7), chloroquine (100 μM) (lane 9), or monensin (30 μM) (lane 10) to the chase medium prevented the trimming of PrP^{Sc}, resulting in a larger M_r of 26–35 kDa. These reagents raise the pH of acidic compartments and thereby inhibit the action of acidic hydrolases. From the results of our studies, we conclude the following: 1) PrP^{Sc} becomes exposed to acidic proteases shortly after its synthesis, 2) acidic vesicular pH is not obligatory for the synthesis of PrP^{Sc}, and 3) PrP^{Sc} synthesis can proceed without the N-terminal degradation. Lysosomotropic amine-sensitive trimming of PrP^{Sc} in cultured cells also has been reported by other investigators (Caughey *et al.*, 1991).

Although demonstrating that at least some PrP^{Sc} is first synthesized as a full-length molecule before being trimmed, the results of Figure 6A do not provide us with a clear view of the kinetics of the process. To study better the course of the N-terminal trimming of PrP^{Sc}, we took advantage of the fact that in cells treated with tunicamycin, PrP^{Sc} synthesis occurs at a more rapid rate and in a stepwise manner (Taraboulos *et al.*, 1990a). ScN_2a cells were labeled for 1 h with 30 μg/ml tunicamycin and then chased for 1, 2, 3, and 4 h and assayed for insoluble PrP as described above (Figure 6B). Protease-resistant PrP^{Sc} was clearly first synthesized as a 26-kDa molecule (lanes 2 and 3), but upon longer chase this was replaced by a 19-kDa species (lanes 4 and 5) identical in its M_r to proteinase K-digested unglycosylated PrP^{Sc}. In contrast, when NH_4Cl (Figure 6B) was included in both the pulse and the chase periods, the 26-kDa band could be detected even after 6 h of chase, and no 19-kDa PrP species was observed. Nevertheless, protease-resistant PrP^{Sc} was still generated (lane 8), reproducing the results obtained above with cells not treated with tunicamycin. PrP^{Sc} synthesized with tunicamycin was trimmed ~1 h after its synthesis. Considering that the labeling period was 1 h long, the sharp definition of the trimming time suggests the coordinated exposure of the radiolabeled PrP^{Sc} molecules to acid proteases.

Incomplete N-Terminal Degradation of PrP^{Sc} in the Brain During Scrapie

Because PrP^{Sc} is stable in ScN_2a and ScHaB cells ($t_{1/2}$ > 24 h [Borchelt *et al.*, 1990; Borchelt *et al.*, 1992]), the vast majority of PrP^{Sc} molecules present in these cells at any given instant should have been already processed in lysosomes and thus lack the protease-sensitive N-terminus. We verified this contention by probing Western blots of cell lysates (Figure 7A, ScN_2a, lanes 4 and 10; ScHaB, lanes 6 and 12) with the synthetic peptide antisera R002 (directed against the peptide P2, the N-terminus of PrP 33–35) (Figure 7A, lanes 1–6) or R013 (directed against the peptide P1, the N-terminus of the protease-resistant core PrP 27–30) (Figure 7A, lanes 7–12). We also analyzed the PrP^{Sc} species present in the lysate of a Syrian hamster brain infected with scrapie prions (lanes 2 and 8). The lysates from uninfected HaB

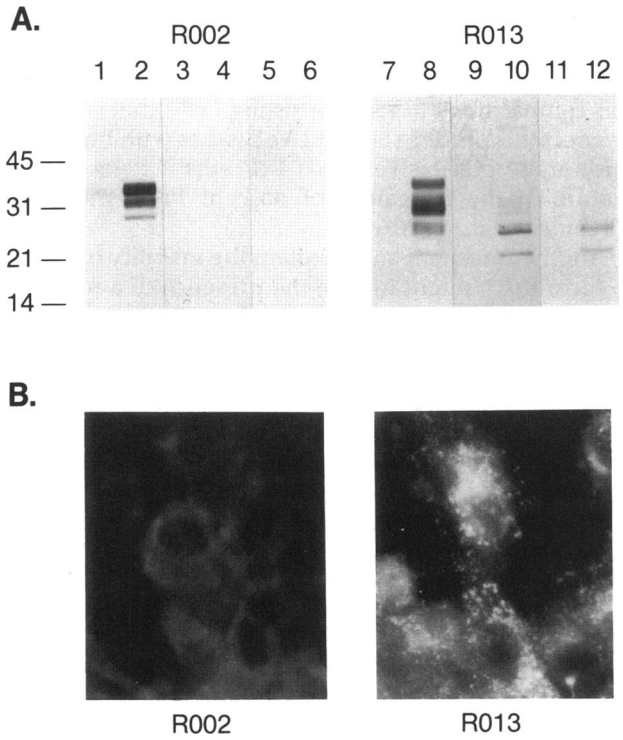

Figure 7. Incomplete N-terminal trimming of PrP^{Sc} in the brain of a Syrian hamster during scrapie. (A) PrP^{Sc} accumulating in hamster brains and in cultured cells was separated from PrP^{C} by ultracentrifugation and analyzed by Western blotting. Lanes 1 and 7, uninfected hamster; lanes 2 and 8, hamster at the final stages of Sc237 scrapie; lanes 3 and 9, N_2a cells; lanes 4 and 10, ScN_2a cells; lanes 5 and 11, HaB cells; lanes 6 and 12, ScHaB cells. The blots were probed with antisera R002 (lanes 1–6) and R013 (lanes 7–12), respectively, directed against PrP-peptides P2 and P1. R002 detected PrP^{Sc} species in the scrapie-infected brain (lane 2) but not in scrapie-infected cells (lanes 4 and 6). (B) ScHaB cells were treated for PrP^{Sc} immunofluorescence using R002 or R013, as indicated. No intracellular PrP^{Sc} was detected with R002.

(lanes 5 and 11) and N_2a (lanes 3 and 9) cells, as well as from the brain of an uninfected Syrian hamster (lanes 1 and 7), were also included. PrP^{Sc} was separated from PrP^{C} by ultracentrifugation (Meyer *et al.*, 1986). Except for a faint band detected in the uninfected brain lysate (~33 kDa, lane 1), no sedimentable PrP was detected from the uninfected samples. As expected, sedimentable PrP^{Sc} from the scrapie-infected cells was recognized by R013 (lanes 10 and 12) but not by R002 (lanes 4 and 6). In contrast, R002 recognized several sedimentable PrP^{Sc} species in the lysate of scrapie hamster brain (Figure 7A, lane 2). R013, however, detected additional PrP^{Sc} species of lower M_r (Figure 7B, lane 8). This indicates that some but not all PrP^{Sc} molecules accumulating in the brain during scrapie lose their N-terminus and demonstrates again that trimming of PrP^{Sc} is not necessary for its synthesis.

Confirming the above results obtained by Western blot analysis, the cytoplasmic accumulation of PrP^{Sc} in ScHaB cells could be easily detected by immunofluorescence using the anti-P1 antiserum R013, but not with anti-P2 antiserum R002 (Figure 7B).

DISCUSSION

From the studies reported here and other investigations, a picture of the cell biology of PrP^{Sc} synthesis is beginning to emerge (Figure 8). PrP^{Sc} is synthesized during a posttranslational process from PrP^{C} or a PrP^{Sc} precursor (Borchelt *et al.*, 1990; Taraboulos *et al.*, 1990a). Studies with BFA indicate that PrP^{Sc} synthesis does not occur in the ER-Golgi and that transport down the secretory pathway is required for this synthesis. Experiments with monensin demonstrate that PrP^{Sc} precursor traverses the mid-Golgi in the same time frame as PrP^{C}. These PrP molecules continue along the secretory pathway to the cell surface where they are bound by a glycosylinositol phospholipid anchor (Caughey and Raymond, 1991; Borchelt *et al.*, 1992). A minority of these PrP molecules are then converted to PrP^{Sc}, presumably either in the endocytic pathway or on the plasma mem-

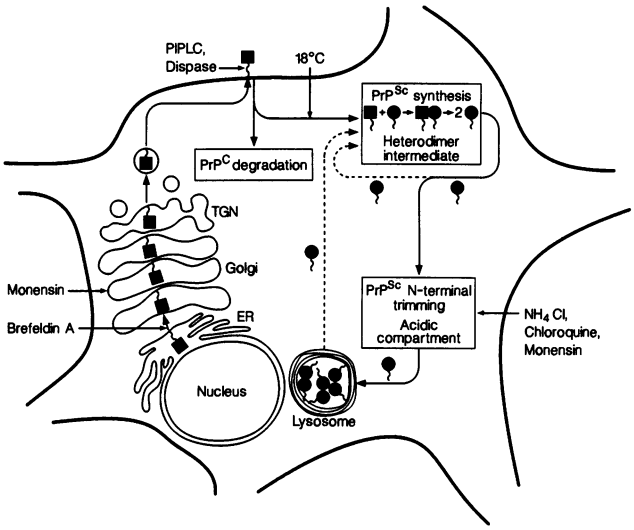

Figure 8. Pathways of prion protein synthesis and degradation in cultured cells. PrP^{Sc} is denoted by circles; squares designate PrP^{C} and the PrP^{Sc} precursor, which may be indistinguishable. Rectangular boxes denote as yet unidentified subcellular compartments. Before becoming protease resistant, the PrP^{Sc} precursor transits through the plasma membrane and is sensitive to dispase or PIPLC added to the medium. PrP^{Sc} synthesis probably occurs in a compartment accessible from the plasma membrane, such as caveolae or endosomes; PrP^{Sc} formation is blocked at 18°C. PrP^{Sc} synthesis probably occurs through the interaction of PrP^{Sc} precursor with existing PrP^{Sc}; the dotted lines denote possible feedback pathways for the reflection of PrP^{Sc} in the active site. Acidic pH within vesicles is not obligatory for PrP^{Sc} synthesis. One to 2 h after PrP^{Sc} formation, it is N-terminally trimmed by an acidic protease; PrP^{Sc} then accumulates primarily in secondary lysosomes. The inhibition of PrP^{Sc} synthesis by BFA demonstrates that the ER-Golgi is not competent for its synthesis and that transport of PrP down the secretory pathway is required for the formation of PrP^{Sc}.

brane. BFA is the first compound found to inhibit the synthesis of PrP^{Sc}.

How Does BFA Prevent the Synthesis of PrP^{Sc}?

Presence of BFA throughout the pulse and the chase periods inhibited the formation of PrP^{Sc}, demonstrating that export from the ER-Golgi is required for the formation of PrP^{Sc}. Although other studies have shown that PrP^{Sc} is likely to be formed after the transit of the polypeptide to the cell surface (Caughey and Raymond, 1991; Borchelt *et al.*, 1992), a causal relationship between transit of the protein along the secretory pathway and PrP^{Sc} synthesis was not established until now. It was possible that the acquisition of protease resistance occurred slowly by a process unrelated to the subcellular localization of PrP, but the kinetics of this process coincided with the export of the PrP^{Sc} precursor to the cell surface. This scenario would have given the appearance that PrP transport is required for the synthesis of PrP^{Sc}. Indeed, the BFA studies reported here show that is not the case, and they demonstrate a causal relationship between PrP transport along the secretory pathway and PrP^{Sc} synthesis.

Several lines of evidence suggest that BFA inhibits PrP^{Sc} synthesis through its action on the secretory pathway rather than by directly interfering with the conversion of PrP^{C} to PrP^{Sc}. First, PrP^{Sc} synthesis was completely blocked only when BFA was present during the pulse and chase periods. When BFA was added only during the chase period, at the time when protease-resistance is acquired, then some PrP^{Sc} was synthesized. Second, the BFA concentration thresholds for PrP^{Sc} inhibition and for blocking PrP^{C} export to the plasma membrane were identical. Moreover, preliminary experiments with forskolin, a competitive antagonist to BFA (Lippincott-Schwartz, 1991), suggest that it reversed the BFA block on protease-resistant PrP synthesis concurrent with the restoration of protein secretion. It seems likely that BFA inhibits the formation of PrP^{Sc} by confining its precursor to the ER-Golgi. It will be interesting to see whether recombinant PrP carrying ER retention signals such as KDEL (Lys-Asp-Glu-Len) (Munro and Pelham, 1987) can acquire protease-resistance in scrapie-infected cells not treated with BFA. The partial inhibition of PrP^{Sc} synthesis by BFA added only to the chase might result from the action of BFA on the early endosomal system (Hunziker *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991).

Why is the ER in BFA-treated cells unable to synthesize PrP^{Sc}? One possibility is that this organelle lacks some essential enzymes or cofactors. Because no scrapie-specific covalent modifications have been detected to date on PrP^{Sc} (Prusiner, 1991; Stahl and Prusiner, 1991), it is unlikely that a missing cofactor functions in covalently modifying PrP^{Sc}. More likely, the conversion of PrP^{C} to PrP^{Sc} involves misfolding, aberrant membrane insertion (Hay *et al.*, 1987), or aggregation with other molecules. It is possible that the ER-Golgi of BFA-treated cells are unable to synthesize de novo PrP^{Sc}, because this compartment lacks pre-existing PrP^{Sc} that is needed to direct this process (Figure 2A). Studies with transgenic mice argue persuasively that PrP^{C}-PrP^{Sc} heterodimers feature in the formation of nascent PrP^{Sc} molecules (Prusiner *et al.*, 1990).

Another mechanism to explain the inability of the ER to form PrP^{Sc} might invoke the presence of a molecule in the ER that actively prevents the synthesis of PrP^{Sc}. For instance, molecular chaperones (Rothman, 1989) or other proteins functioning in the proper folding of polypeptides could prevent a putative conformational change involved in the formation of PrP^{Sc}. Further investigations are necessary to distinguish among these possible mechanisms.

Interestingly, the formation of PrP^{Sc} increased in ScN_2a cells when they were exposed to subinhibitory levels of BFA throughout the pulse and the chase period (Figure 3). The basis for this phenomenon is unclear. It is possible that aberrantly glycosylated PrP synthesized at these BFA concentrations (Figure 3A, lane 5) is preferentially converted to PrP^{Sc}. An even more pronounced increase in PrP^{Sc} synthesis is observed when cells are treated with tunicamycin during the radiolabeling period (Taraboulos *et al.*, 1990a).

N-Terminal Trimming of Mature PrP^{Sc}

That the N-terminus of PrP^{Sc} undergoes a lysosomotropic amine-sensitive trimming shortly after the synthesis of protease-resistant PrP^{Sc} is consistent with the ultrastructural detection of PrP^{Sc} in acid phosphatase-positive vesicles in ScHaB and ScN_2a cells (McKinley *et al.*, 1991b). N-terminal truncation of PrP^{Sc} also has been observed in ScN_2a cells by other investigators (Caughey *et al.*, 1991). To assess the kinetics of the N-terminal trimming, we used tunicamycin to reduce the M_r heterogeneity of PrP as well as to accelerate PrP^{Sc} synthesis. We found that the truncated 19-kDa band appeared ~1 h after the acquisition of protease resistance. Whether this trimming occurs in lysosomes or another compartment in the endosomal system is unclear. Diment and colleagues (1988) have provided evidence for the presence of lysosomal hydrolases in endosomal compartments. Our results also indicate that the lysosomal trimming is not an obligatory step in PrP^{Sc} synthesis; instead, the lysosomal accumulation of PrP^{Sc} may reflect ineffective attempts of cells to degrade unwanted proteins.

Because the degradation of PrP^{C} appears to be mainly nonlysosomal (Taraboulos, unpublished data), PrP^{Sc} must acquire some sorting signal that directs it to secondary lysosomes. Deciphering the nature of such a signal may point to the structural difference between the PrP isoforms. One possibility is that PrP^{Sc} aggre-

gation causes its endocytosis. Aggregated PrP^{Sc} in purified prion preparation is indeed rapidly phagocytosed by N_2a and HaB cells (Taraboulos *et al.*, 1990b). However, aggregates of PrP^{Sc}, such as prion rods, have not been documented in vivo in cultured cells (McKinley *et al.*, 1991a).

Where is PrP^{Sc} Formed?

Whether PrP^{Sc} synthesis occurs in a specific subcellular compartment or in many different organelles is unclear (Figure 8). The inhibition of PrP^{Sc} formation by BFA demonstrates that not every compartment is competent to support PrP^{Sc} synthesis. The kinetics of PrP^{Sc} synthesis in ScHaB cells (Borchelt *et al.*, 1992), as well as in ScN_2a cells treated with tunicamycin (Taraboulos *et al.*, 1990a), suggest that newly synthesized PrP molecules all arrive in a coordinated fashion at a specific location where they are converted to PrP^{Sc}. Identifying this putative compartment will be important because it may reveal the nature of the posttranslational event that features in the synthesis of PrP^{Sc}.

The studies reported here indicate that PrP^{Sc} synthesis occurs somewhere between the Golgi complex and lysosomes. Other investigations argue that PrP continues along the secretory pathway to the cell surface before its conversion to PrP^{Sc}. Kinetic studies show that PrP^{C} transits to cell surface rapidly (<1 h) before detection of the first PrP^{Sc} molecules. Under some experimental conditions, release of PrP from the plasma membrane by PIPLC (Caughey and Raymond, 1991) or dispase (Borchelt *et al.*, 1992) prevents PrP^{Sc} synthesis. These results contend that the synthesis of PrP^{Sc} occurs in a subcellular compartment located between the plasma membrane and secondary lysosomes. The route of PrP internalization may involve the nonclathrin coated vesicles or the 'caveolae' as recently described for another GPI-anchored protein, the folate receptor (Rothberg *et al.*, 1990).

Because existing PrP^{Sc} molecules probably feature directly in the synthesis of nascent PrP^{Sc} molecules, as indicated by studies with transgenic mice (Prusiner *et al.*, 1990), the PrP^{Sc} synthesis pathway must enable PrP^{C} or a precursor to interact with existing PrP^{Sc}. What subcellular compartments would permit the PrP^{Sc} precursor to meet pre-existing PrP^{Sc}? The endosomal system is certainly a candidate, because a major portion of intracellular PrP^{Sc} accumulates in secondary lysosomes (McKinley *et al.*, 1991b). Recent results on the action of BFA on lysosomes (Lippincott-Schwartz *et al.*, 1991) raise the possibility that some transport out of these organelles may occur. The action of BFA on the endocytic pathway (Hunziker *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991) may reflect in the partial inhibition of PrP^{Sc} synthesis that was observed when the drug was added during the chase period. In addition, our ultrastructural studies (McKinley *et al.*, 1991b) did not rule out the presence of PrP^{Sc} in the TGN and endosomes as these organelles could not be identified in those studies, or on the plasma membrane where it was not possible to differentiate between PrP^{Sc} and PrP^{C}. Thus, alternative pathways for PrP^{Sc} synthesis might include the delivery of endocytosed PrP^{C} to a subcompartment of the Golgi apparatus where it would be converted to PrP^{Sc} (Snider and Rogers, 1985; Green and Kelly, 1991) or the synthesis of PrP^{Sc} on the plasma membrane followed by its endocytosis (Figure 8).

Transit of PrP through the plasma membrane may not be obligatory for the synthesis of PrP^{Sc}. Scrapie-infected cells treated with tunicamycin, an inhibitor of Asn-linked glycosylation, do not seem to transport PrP efficiently to the plasma membrane (Caughey *et al.*, 1990; Rogers *et al.*, 1990), yet PrP^{Sc} is synthesized with high efficiency (Taraboulos *et al.*, 1990a). If PrP^{Sc} synthesis occurs in the endocytic pathway, then autophagy (Gordon and Seglen, 1988), for example, could offer an intracellular route for PrP to reach this compartment in cells treated with tunicamycin.

The discovery that BFA effectively inhibits PrP^{Sc} synthesis in cultured cells raises the possibility that this drug might prevent or slow the course of scrapie in animals as well as experimental Gerstmann-Sträussler-Scheinker disease in transgenic mice (Hsiao *et al.*, 1990). If BFA can be administered chronically to rodents to prevent experimental prion diseases, then it might prove efficacious in humans. Interestingly, PrP^{C} does not seem to be an essential protein because mice with disrupted PrP genes are apparently normal at >11 mo of age (Büeler *et al.*, 1992). Because individuals at risk for the inherited prion diseases can now be identified decades in advance of the onset of illness (Goldgaber *et al.*, 1989; Hsiao *et al.*, 1989, 1991; Owen *et al.*, 1989; Goldfarb *et al.*, 1991; Prusiner, 1991), an effective pharmacological therapy that inhibits PrP^{Sc} synthesis might significantly delay the appearance of neurologic dysfunction.

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