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Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation

(prion diseases/site-directed mutagenesis/tunicamycin/complex type oligosaccharides/posttranslational modification)

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ABSTRACT **The scrapie and cellular isoforms of the prion protein (PrP^{Sc} and PrP^{C}) differ strikingly in a number of their biochemical and metabolic properties. The structural features underlying these differences are unknown, but they are thought to result from a posttranslational process. Both PrP isoforms contain complex type oligosaccharides, raising the possibility that differences in the asparagine-linked glycosylation account for the properties that distinguish PrP^{C} and PrP^{Sc}. ScN_2a and ScHaB cells in culture produce several PrP molecules with relative molecular masses of 26–35 kDa and proteinase K-resistant cores of 19–29 kDa. When the cells were treated with tunicamycin, this heterogeneity was eliminated and a single PrP species of 26 kDa was observed. Several hours after its synthesis, a fraction of this protein became insoluble in detergents and acquired a proteinase K-resistant core, thus displaying two of the biochemical hallmarks of PrP^{Sc}. Synthesis in the presence of tunicamycin restricted the proteinase K-resistant cores of PrP to a single species of 19 kDa. No proteinase K-resistant PrP was found in uninfected cells. Expression of a mutated PrP gene lacking both asparagine-linked glycosylation sites in ScN_2a cells resulted in the synthesis of 19-kDa proteinase K-resistant PrP molecules. We conclude that asparagine-linked glycosylation is not essential for the synthesis of proteinase K-resistant PrP and that structural differences unrelated to asparagine-linked oligosaccharides must exist between PrP^{C} and PrP^{Sc}. Whether unglycosylated PrP^{Sc} molecules are associated with scrapie prion infectivity remains to be established.**

The scrapie prion protein (PrP^{Sc}) is an abnormal isoform of the membrane glycoprotein PrP^{C} (1). PrP^{Sc} accumulates in the brains of humans and animals with prion diseases (2–5) and in cultured cells persistently infected with scrapie or Creutzfeldt–Jakob disease prions (6, 7). Much evidence argues that PrP^{Sc} is a major and necessary component of the infectious prion particle (8, 9). The two PrP isoforms differ in many respects: (*i*) PrP^{C} is sensitive to proteolysis, while PrP^{Sc} possesses a protease-resistant core, PrP 27–30 (1); (*ii*) PrP^{C} is soluble in detergents, whereas both PrP^{Sc} and PrP 27–30 form insoluble aggregates in the presence of detergents (10); (*iii*) PrP^{C} is released by digestion with phosphatidylinositol phospholipase C but PrP^{Sc} is not (11, 12); (*iv*) PrP^{C} is found on the cell surface, while PrP^{Sc} is found primarily in the cytoplasm (7); (*v*) PrP^{Sc} immunoreactivity is enhanced by denaturation but PrP^{C} is not (13); (*vi*) PrP^{C} synthesis and degradation are rapid in contrast to that of PrP^{Sc}, which is produced slowly (14, 15).

To date, no structural differences have been found that distinguish PrP^{C} from PrP^{Sc}; it has been suggested that they differ in either chemical or conformational posttranslational modifications (15–17). The PrP isoforms are extensively modified posttranslationally: (*i*) an N-terminal signal is cleaved (1, 18); (*ii*) there is an intramolecular disulfide bond (16); (*iii*) an inositol glycophospholipid anchor residue is added to the C terminus and a C-terminal hydrophobic region is removed (11); (*iv*) the protein is extensively asparagine glycosylated (19–21).

It has been proposed that asparagine-linked oligosaccharides may contain the structural difference between PrP^{C} and PrP^{Sc} (17). Cell cultures supporting replication of prions and synthesis of PrP^{Sc} provide a system to test this hypothesis. We show here that ScN_2a (6) and ScHaB cells (7) synthesize, when treated with tunicamycin, a detergent-insoluble 26-kDa PrP species containing a 19-kDa proteinase K-resistant core. No such protease-resistant PrP was detected in uninfected cells. Furthermore, removal of the two asparagine-linked glycosylation sites of the PrP gene open reading frame (ORF) by *in situ* mutagenesis similarly resulted in the synthesis of a proteinase K-resistant unglycosylated PrP species in ScN_2a cells. We conclude that asparagine-linked glycosylation is not necessary for the synthesis of PrP^{Sc} and thus structural differences must exist between the PrP isoforms that are unrelated to asparagine-linked carbohydrates.

MATERIALS AND METHODS

Reagents and Cells. Reagents were of the highest grades commercially available. Tunicamycin and swainsonine were from Boehringer Mannheim. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase^F, [PNGase F; asparagine-linked-oligosaccharide-glycopeptide-N^4-(*N*-acetyl-β-D-glucosaminyl)-L-asparagine amidohydrolase, EC 3.5.1.52 was a generous gift from A. Tarentino (Albany, NY). ScN_2a is a persistently infected subclone of N_2a (6) and ScHaB-4-C4 is a subclone of HaB (7). Cells were grown at 37.5°C in Dulbecco's minimal essential (DME) H-16 medium supplemented with 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (FCS). Cells transfected with the pSPOX·neo constructions were grown in 1 mg of G418 per ml.

Antibodies. R017 was raised in a rabbit against SDS-denatured prion hamster rods (15, 22). R073 was raised in a rabbit against SDS/PAGE purified hamster PrP 27–30 (13). R013, R030, and R009 are monospecific rabbit antisera raised against the hamster PrP peptides P1, P5, and P3, respectively (23), which correspond to amino acids 90–102, 142–174, and 220–233, respectively (17). Mouse monoclonal antibody 3F4 was a gift from R. Kascsak (Staten Island, NY) (24).

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Abbreviations: PrP, prion protein; PrP^{Sc}, scrapie isoform of PrP; PrP^{C}, cellular isoform of PrP; PMSF, phenylmethylsulfonyl fluoride; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; ORF, open reading frame; NP-40; Nonidet P-40.

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Radioactive Labeling and Inhibitors. Confluent cells growing in 10-cm plates were rinsed three times with phosphate-buffered saline (PBS) and incubated for 30 min with methionine- and cysteine-free DME-16 medium (MCF-DME) with 5% FCS. The medium was then replaced with MCF-DME and 5% FCS supplemented with 0.5 mCi of Tran^{35}S-label per ml (ICN). After the appropriate labeling period, the cells were rinsed twice with DME and then either immediately lysed or chased in regular DME containing 10% FCS for a suitable time. Tunicamycin or swainsonine was added to the medium in both the starving and the labeling periods. Both inhibitors were dissolved in dimethyl sulfoxide. Tunicamycin was used at a final concentration of 30 μg/ml in N_2a cells and at 5 μg/ml in HaB cells; swainsonine was used at 5 μg/ml.

Cell Lysis and Proteinase K Digestion. Cells growing on 10-cm plates were rinsed twice with ice-cold PBS and then lysed *in situ* by the addition of 3 ml of cold lysis buffer [100 mM NaCl/10 mM EDTA/0.5% Nonidet P-40 (NP-40)/0.5% sodium deoxycholate/10 mM Tris·HCl, pH 7.8] containing 0.1 unit of aprotinin per ml and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). (The protease inhibitors were omitted if the lysates were to be treated with proteinase K.) The insoluble material was removed by a low-speed spin (5 min at 1000 × *g*). The proteins in the supernatants were then precipitated by the addition of 12 ml of methanol at −20°C. For proteinase K digestion, the protease was added to the low-speed supernatant to a final concentration of 20 μg/ml and proteolysis was performed for 1 hr at 37°C. The reaction was stopped by the addition of PMSF to a final concentration of 3 mM.

Immunoprecipitation of PrP. The methanol pellet from the cell lysate was resuspended in 1 ml of 3 M guanidine thiocyanate (GdnSCN)/20 mM Tris·HCl, pH 7.8, incubated for 10 min at room temperature, and the proteins were again precipitated by the addition of 4 ml of methanol. GdnSCN enhanced PrP^{Sc} immunobinding (13). Pellets were resuspended in 2 ml of TNS [100 mM NaCl/1% sodium sarcosinate (Sarkosyl)/10 mM Tris·HCl, pH 7.8, supplemented with 0.1 mM PMSF and 0.1 unit of aprotinin per ml]. Two microliters of the appropriate antiserum was then added and the lysates were incubated for 12–18 hr at 4°C. The immunocomplexes were recovered by using protein A-Sepharose. For immunoprecipitation of whole cell PrP^{Sc} (see Fig. 4*A*), cells were lysed on the plate in 1.5 ml of 1% NP-40/1 mM EDTA/10 mM Tris·HCl, pH 7.8, containing 30 μg of proteinase K per ml. Proteolysis was performed for 1 hr at 37°C and was stopped by the addition of PMSF to 3 mM for 30 min. Then 1.5 ml of 6 M GdnSCN and 2% Sarkosyl were added. The lysate was transferred to a glass tube and sonicated in a bath sonicator for 20 sec to reduce its viscosity. After precipitation with methanol, PrP was immunoprecipitated as described above.

PAGE, Fluorography, and PNGase F Digestion. Proteins were separated by electrophoresis according to Laemmli (25). The fluor "Amplify" (Amersham) was used for radiofluorography. For Western blotting, the proteins were transferred to a nitrocellulose membrane (26) and were detected by the ProtoBlot detection system (Promega Biotec). Immunoprecipitated PrP was removed from the protein A-Sepharose by boiling in 50 μl of 0.1% SDS/30 mM Tris·HCl, pH 8.5, for 10 min. After the addition of NP-40 to a concentration of 1% and 0.5 milliunit of PNGase F, the samples were digested for 18 hr at room temperature.

Construction of the Mutagenized PrP Gene. The recombinant mouse PrP ORF designated MHM2 (M.S., unpublished data; also see text) served as the basis for construction of the glycosylation mutant. MHM2 contains an epitope recognized by the hamster-specific monoclonal antibody 3F4 (24). This substitution does not disrupt its ability to form proteinase K-resistant PrP in ScN_2a cells (M.S., unpublished data). To construct the double-site glycosylation mutant $MHM2_{Ala182/198}$, the two asparagine-linked glycosylation sites were abolished by substituting alanine residues for the tyrosines at positions 182 and 198 (27). Both the wild-type and the mutant MHM2 ORF were expressed in ScN_2a cells when the mammalian expression plasmid pSPOX·neo was used (M.S., unpublished data). pSPOX·neo carries a gene conferring resistance to the antibiotic G418.

RESULTS

Tunicamycin-Treated Cells Synthesize Proteinase K-Resistant PrP. Both ScN_2a (6, 14, 15) and ScHaB cells (7) synthesize PrP molecules that yield, upon digestion with proteinase K, three distinct bands of 19, 25, and 27–30 kDa. These bands all react with monospecific antibodies directed against both N-terminal (P1) and C-terminal (P3) regions of PrP 27–30 (15). HaB (Fig. 1*A*, lanes 1, 2, 5, and 6), ScHaB (lanes 3, 4, 7, and 8), N_2a (lanes 9 and 10), and ScN_2a (lanes 11 and 12) cells were labeled with Tran^{35}S-label for 2 hr in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of tunicamycin (30 μg/ml for N_2a cells and 3 μg/ml for HaB cells). The PrP molecules synthesized during the labeling period were then immunoprecipitated from the cell lysates (lanes 1–4) without proteolytic treatment and analyzed by

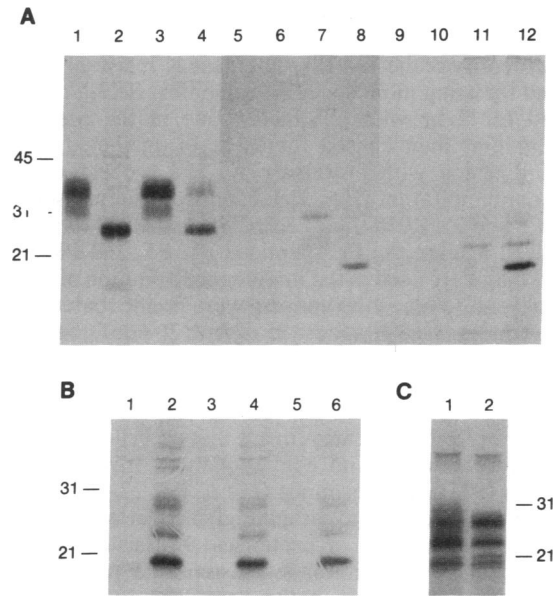

FIG. 1. Protease-resistant PrP^{Sc} is synthesized by scrapie-infected cells in the presence of tunicamycin. (*A*) Scrapie-infected (lanes 3, 4, 7, 8, 11, and 12) and control noninfected (lanes 1, 2, 5, 6, 9, and 10) cells were labeled for 2 hr with [^{35}S]methionine (lanes 1–8, HaB cells; lanes 9–12, N_2a cells). In lanes 2, 4, 6, 8, 10, and 12 tunicamycin was added to the labeling medium. For PrP^{C} analysis (lanes 1–4) the cells were lysed at the end of the labeling period and processed for PrP immunoprecipitation. For analysis of PrP^{Sc} (lanes 5–12), the cells were further chased in unlabeled medium for 16 hr, lysed, and digested with proteinase K (20 μg/ml for 1 hr at 37°C) before immunoprecipitation. The immunopurified PrP was analyzed by SDS/PAGE and detected by autoradiography. The rabbit antiserum R017, raised against SDS-denatured purified hamster prions, was used in the immunoprecipitation. (*B*) PrP^{Sc} synthesized in ScN_2a cells exposed to tunicamycin was immunoprecipitated by using the monospecific peptide antisera R013 (lanes 1 and 2), R030 (lanes 3 and 4), and R013 (lanes 5 and 6). R013, R030, and R009 were raised against the PrP peptides P1, P5, and P3, respectively. Preadsorption of the antisera with the corresponding synthetic free peptides (25 μg/ml; 30 min) before their use strongly decreased the intensity of the 19-kDa band, demonstrating its PrP identity (lanes 1, 3, and 5). (*C*) ScN_2a cells were radiolabeled for 2 hr in the presence of swainsonine (5 μg/ml; lane 2) or without any inhibitor (lane 1). After an 18-hr chase the proteinase K-resistant PrP was immunoprecipitated. Exposure to swainsonine did not prevent PrP^{Sc} synthesis but resulted in the sharpening of the 27- to 30-kDa band.

PAGE and autoradiography. Since previous studies have shown that resistance to proteolysis is acquired by PrP^{Sc} posttranslationally (15), some cells were further chased in unlabeled medium for 16 hr (lanes 5–12). In this case, the cell lysate was subjected to limited proteolysis catalyzed by proteinase K prior to PrP immunoprecipitation and analysis.

In both normal and scrapie-infected HaB cells, PrP assumed its characteristic 26- to 35-kDa heterogeneous pattern in the cells not treated with tunicamycin (28) (lanes 1 and 3) while the 26-kDa species became prominent when the labeling was performed in the presence of the inhibitor (lanes 2 and 4), as has been reported for PrP^{C} in N_2a cells (14). No proteinase K-resistant PrP was detected in the uninfected HaB (lanes 5 and 6) or N_2a cells (lanes 9 and 10) after a 16-hr chase, even when tunicamycin was present during radiolabeling. In both ScHaB (lane 7) and ScN_2a (lane 11) cells not treated with tunicamycin, the characteristic proteinase K-resistant PrP^{Sc} bands appeared. When biosynthesis was performed in the presence of tunicamycin, the heterogeneous pattern of PrP in both cell lines (lanes 8 and 12) was reduced almost completely to a 19-kDa band after limited proteolysis, identical in its apparent molecular mass to the lower PrP band synthesized in the untreated cells (lanes 7 and 11). Previous studies have indeed shown that deglycosylation of PrP 27–30 reduces its size to 19 kDa (21).

The identity of the 19-kDa proteinase K-resistant band was verified by using monospecific antibodies. ScN_2a cells were labeled for 2 hr with [^{35}S]methionine in the presence of tunicamycin, then chased for 16 hr in unlabeled medium, lysed, digested with proteinase K, and processed for PrP immunoprecipitation (Fig. 1*B*). Monospecific antisera R013 (lanes 1 and 2), R030 (lanes 3 and 4), and R009 (lanes 5 and 6) (raised against the PrP peptides P1, P5, and P3, respectively) (23) were used in the immunoprecipitation of PrP from ScN_2a lysates. When the antisera were preincubated with the corresponding free peptides (25 μg/ml; 30 min), the intensity of the resulting 19-kDa band was strongly reduced (Fig. 1*B*; lanes 1, 3, and 5). These results demonstrate that the proteinase K-resistant 19-kDa species immunoprecipitated from cells treated with tunicamycin are indeed PrP molecules and that they contain amino acid sequences from both the N terminus (P1) and C terminus (P3) of PrP 27–30.

Other inhibitors of asparagine glycosylation also did not prevent the synthesis of proteinase K-resistant PrP^{Sc}; the α-mannosidase II inhibitor swainsonine (5 μg/ml) (29, 30) resulted in a reduction in the heterogeneity of the 27- to 30-kDa band (Fig. 1*C*, lane 2) obtained after proteolysis of the cell lysate. Glycoproteins of cells treated with swainsonine possess hybrid type oligosaccharides (31).

The 19-kDa Proteinase K-Resistant PrP Species Are Insoluble in Detergent and Are Unglycosylated. Cells were labeled for 2 hr and then either directly lysed with lysis buffer (Fig. 2*A*, lanes 1–4) or chased for an additional 16 hr in unlabeled medium and then lysed (lanes 5–8). In the latter case, the lysates were digested with proteinase K. All lysates were subjected to high-speed centrifugation (100,000 × *g* for 1 hr at 4°C) and the PrP was recovered from both supernatants (lanes 1, 3, 5, and 7) and pellets (lanes 2, 4, 6, and 8) by immunoprecipitation. None of the proteinase K-resistant PrP species synthesized in ScN_2a cells in the absence (lanes 5 and 6) or presence (lanes 7 and 8) of tunicamycin were soluble in the lysis buffer. Thus, the 19-kDa unglycosylated PrP^{Sc} species (Fig. 2*A*, lane 8) displays the detergent insolubility characteristic of PrP^{Sc}.

The lack of asparagine-linked carbohydrates on the 19-kDa proteinase K-resistant PrP molecules synthesized in tunicamycin-treated cells was confirmed by digestion with the enzyme PNGase F (32, 33) (Fig. 2*B*). Cells were radiolabeled in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of tunicamycin. The proteinase K-resistant PrP cores were

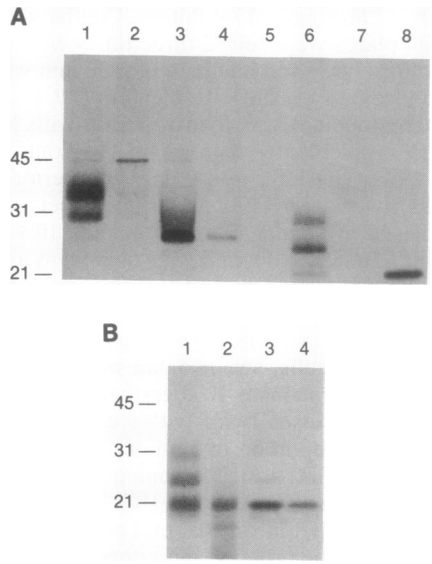

FIG. 2. The 19-kDa proteinase K-resistant PrP species are insoluble in detergent and are unglycosylated. (*A*) ScN_2a cells were labeled with [^{35}S]methionine for 2 hr in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of tunicamycin (30 μg/ml). The cells were then either lysed immediately in lysis buffer (lanes 1–4) or chased for 16 hr in unlabeled medium before lysis (lanes 5–8). The insoluble material was pelleted by low-speed centrifugation. For lanes 5–8, the lysate was then digested with proteinase K (20 μg/ml for 1 hr at 37°C). The lysates were then subjected to a high-speed centrifugation (100,000 × *g* for 1 hr at 4°C). PMSF was then added to the supernatants and the pellets, and these fractions were then processed for PrP immunoprecipitation with antiserum R017 (supernatants, lanes 1, 3, 5, and 7; pellets, lanes 2, 4, 6, and 8). Note that the proteinase K-resistant PrP species [27–30, 25, and 19 kDa in cells not treated with tunicamycin (lanes 5 and 6) and 19 kDa in cells treated with the inhibitor (lanes 7 and 8)] are completely insoluble in detergents, hence displaying another biochemical hallmark of PrP^{Sc}. (*B*) ScN_2a cells were labeled for 2 hr in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of tunicamycin. After a 16-hr chase, they were lysed, digested with proteinase K, and PrP was immunoprecipitated by using the R017 antiserum. In lanes 2 and 4, the immunopurified PrP was further digested with PNGase (0.5 milliunit/ml; 18 hr). The resulting PrP was then analyzed by SDS/PAGE. Note that the higher molecular mass PrP^{Sc} bands in lane 1 are reduced to the 19-kDa band by the PNGase digestion (lane 2), while the 19-kDa band is left unchanged by this glycosidase (lanes 2 and 4).

immunoprecipitated and then digested with PNGase F (lanes 2 and 4). After digestion with the glycosidase, the higher molecular mass bands (30 and 27 kDa) disappeared, while the 19-kDa band was reinforced in untreated cells. The 15- to 16-kDa band appearing in lane 2 is presumably a degradation product due to trace protease activity in the PNGase F preparations (33). This protease apparently cleaves the PrP peptide at the N-terminal side of Asp-177 (N. Stahl, personal communication), resulting in a fragment with a predicted mass of ≈15 kDa. In the tunicamycin-treated cells, the 19-kDa band was unchanged by digestion with PNGase F. This confirms that the 19-kDa PrP species indeed lacks asparagine-linked oligosaccharides.

PrP Gene Lacking Asparagine-Linked Glycosylation Sites Directs Synthesis of 19-kDa Proteinase K-Resistant PrP. To distinguish products of a mutated gene from endogenous PrP^{Sc}, we utilized the recombinant PrP sequence MHM2 (M.S., unpublished data) comprising the mouse PrP ORF (34) in which Leu-108 and Val-111 were replaced by methionines (Fig. 3*A*). These mutations introduce an epitope recognized by the monoclonal antibody 3F4 (M.S., unpublished data), which does not react with the mouse PrP (24). The inclusion of this epitope does not impair the ability of MHM2 PrP to form protease-resistant PrP^{Sc} in ScN_2a cells (M.S., unpublished

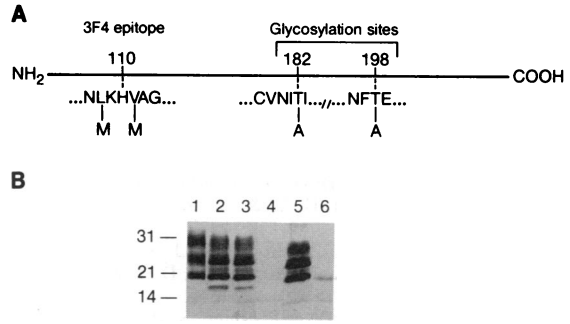

FIG. 3. Mutated PrP gene lacking both asparagine-linked glycosylation sites directs synthesis of proteinase K-resistant PrP in ScN_2a cells. (*A*) Description of the $MHM2_{Ala182/198}$ mutant gene. (*B*) Untransfected ScN_2a cells (lanes 1 and 4) and pooled ScN_2a cells transfected with either the wild-type MHM2 gene (carrying the epitope for recognition by 3F4; lanes 2 and 5) or the corresponding glycosylation mutant $MHM2_{Ala182/188}$ (lanes 3 and 6) were analyzed for proteinase K-resistant PrP. Lysates from cells growing on T25 flasks were loaded on each lane. The lysates were digested with proteinase K (20 μg/ml at 37°C for 1 hr) before boiling in SDS-loading buffer. The Western blots were probed with the PrP polyclonal antiserum R073 (recognizing both hamster and mouse PrP; lanes 1–3) or the hamster-specific 3F4 antibody (lanes 4–6). Note the distinct 19-kDa band detected by 3F4 in the glycosylation mutant (lane 6).

data), thereby allowing the recombinant products to be discriminated from the endogenous mouse PrP^C and PrP^{Sc}.

Both wild-type MHM2 and the glycosylation mutant designated $MHM2_{Ala182/198}$ were introduced into the expression vector pSPOX·neo (M.S., unpublished data) and transfected into ScN_2a cells. We analyzed pooled transfected cells for proteinase K-resistant PrP; untransfected ScN_2a served as controls (Fig. 3*B*). Cell lysates were digested with proteinase K and analyzed by Western blotting using R073 (a polyclonal antibody reacting with both mouse and hamster PrP; lanes 1–3) and 3F4 (lanes 4–6) as probes. As expected, the untransfected ScN_2a (lane 4) did not react with the hamster-specific monoclonal antibody 3F4, while both transfected cells showed characteristic PrP bands with this antibody. The MHM2 gene directed the synthesis of the characteristic proteinase K-resistant PrP triplet (lane 5), whereas cells transfected with the glycosylation mutant $MHM2_{Ala182/198}$ produced a 19-kDa band (lane 6) that was the only 3F4-reactive species. This band has the same molecular mass as the protease-resistant PrP^{Sc} core synthesized in the presence of tunicamycin (Fig. 1). These results confirm that unglycosylated PrP can indeed acquire proteinase K resistance.

The glycosylation mutant reproducibly yielded lower levels of proteinase K-resistant PrP than the wild-type MHM2 gene (M.R., unpublished results). This may be due to inherent low yields of proteinase K-resistant acquisition (e.g., caused by the amino acid substitution) or to unfavorable competition with the resident wild-type mouse PrP for hypothetical PrP^{Sc} synthesis sites. Alternatively, lower transfection efficiencies with the mutant MHM2 may be invoked.

Resistance to Proteolysis Is Acquired Posttranslationally. Previous experiments have shown that in ScN_2a cells, proteinase K-resistant PrP species appear in lysis buffer extracts as a result of a slow, posttranslational process (15). Two possible mechanisms can be invoked to explain this phenomenon: (*i*) proteinase K-sensitive precursors of PrP^{Sc} slowly become resistant to proteolysis; (*ii*) proteinase K-resistant PrP^{Sc} is rapidly synthesized in the cell, but it cannot be extracted in lysis buffer until after a long chase incubation. To differentiate between these possibilities, we performed a pulse–chase experiment in which a modified lysis technique was used, ensuring PrP immunoprecipitation from all subcellular components (Fig. 4*A*).

Both in tunicamycin-treated ScN_2a cells and in untreated cells the proteinase K-resistant PrP species could be detected following the chase (Fig. 4*A*, lanes 3 and 4) but were almost absent from the whole cell lysate at the end of the pulse labeling (lanes 1 and 2). Therefore, we conclude that the resistance to proteolysis is acquired by PrP^{Sc} as a slow posttranslational process, and this process occurs whether PrP possesses or lacks asparagine-linked carbohydrates.

In ScN_2a cells untreated with tunicamycin, PrP^{Sc} becomes resistant to proteinase K-catalyzed proteolysis with a $t_{1/2}$ of ≈15 hr (15). Interestingly, the degradation of PrP^C in both uninfected and scrapie-infected N_2a cells occurs over a similar time interval ($t_{1/2}$, ≈6 hr) (14, 15). We analyzed the stability of PrP^C in N_2a cells and the kinetics for PrP^{Sc} formation in ScN_2a cells after radiolabeling in the presence of tunicamycin. We found (Fig. 4*B*) that unglycosylated PrP^C indeed was much less stable than its glycosylated counterparts ($t_{1/2}$, <2 hr). Moreover, the unglycosylated 19-kDa PrP^{Sc} also acquired resistance to proteinase K-catalyzed degradation faster than the asparagine-glycosylated PrP^{Sc} species in cells not treated with tunicamycin ($t_{1/2}$, ≈3 hr) (Fig. 4*C*). The more rapid conversion of unglycosylated PrP to proteinase K-resistant PrP^{Sc} could be either a direct consequence of its lack of glycosylation or an indirect result of its aberrant subcellular localization (14, 27).

DISCUSSION

The structural features underlying the striking metabolic (15) and biochemical differences between the PrP isoforms are

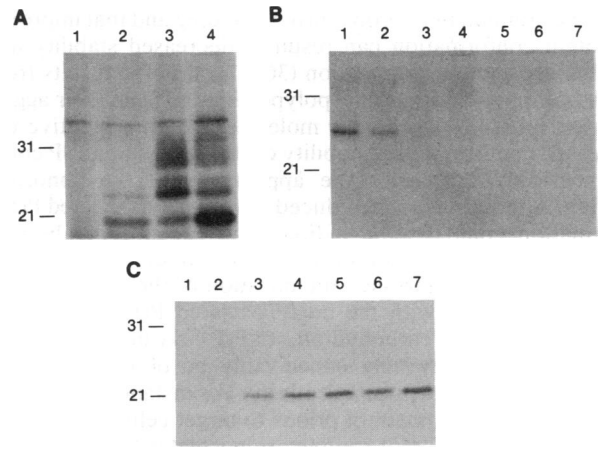

FIG. 4 Kinetics of PrP^{Sc} synthesis and of PrP^C degradation in tunicamycin-treated cells. (*A*) Analysis of the whole cell lysates. ScN_2a cells were labeled with [^{35}S]methionine for 1 hr in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of tunicamycin. In lanes 2 and 4, the cells were further chased in unlabeled medium for 16 hr. The cells were then lysed in a buffer containing 1% NP-40 and 30 μg of proteinase K per ml and digested for 1 hr at 37°C. Thirty minutes after the addition of PMSF (3 mM), the lysed monolayers were solubilized with GdnSCN and Sarkosyl (respective final concentrations, 3 M and 1%) and the viscosity of the solution was reduced by sonication. Proteins were precipitated with 4 vol of methanol; PrP was then immunoprecipitated with the antiserum R017. This solubilization method ensures access to all the cellular proteins and does not restrict the analysis to lysis buffer-soluble proteins alone. The results show that the acquisition of proteinase K resistance by both asparagine-glycosylated and unglycosylated PrP^{Sc} is a slow posttranslational process. (*B* and *C*) N_2a (*B*) and ScN_2a (*C*) cells were labeled for 75 min with [^{35}S]methionine in the presence of 30 μg of tunicamycin per ml. The medium was then replaced by unlabeled medium without tunicamycin and the cells were chased for 0 hr (lane 1), 1 hr (lane 2), 2 hr (lane 3), 3 hr (lane 4), 4 hr (lane 5), 6 hr (lane 6), and 12 hr (lane 7). At the completion of the chase period, the cells were lysed and the PrP was immunoprecipated with R017 antiserum. For the analysis of PrP^{Sc} in *C*, the lysates were digested with proteinase K (20 μg/ml for 1 hr at 37°C) before immunoprecipitation.

still unknown (35). Both proteins are extensively glycosylated (19, 21). The diversity of carbohydrate structures could yield >400 different forms of the prion protein (20). Because of this complexity and the fact that the asparagine-linked carbohydrates constitute up to 30% of the molecular mass of PrP 27–30, it was suggested that they may harbor the critical information differentiating PrP^{Sc} from PrP^{C} (17, 35). Our results argue against this hypothesis, as they demonstrate that two important biochemical hallmarks of PrP^{Sc}, the resistance to proteolysis and the insolubility in detergents (1, 10), can be acquired by unglycosylated PrP (Figs. 1 and 2). Thus, the two PrP isoforms must differ in some other structural feature that is acquired posttranslationally.

The subcellular compartments in which the conversion of PrP^{C} or a precursor into PrP^{Sc} takes place are unknown. PrP^{Sc} accumulates primarily within the interior of cells (7), while fully modified PrP^{C} localizes to the plasma membrane as early as 20 min after the translation (14). Since unglycosylated PrP largely appears to remain in the interior of the cell (27), it seems likely that transformation of PrP molecules into protease-resistant PrP^{Sc} probably takes place intracellularly, suggesting that transit to the plasma membrane is not essential for PrP^{Sc} synthesis. Less probable, the acquisition of resistance to proteolysis by PrP^{Sc} may occur either on the plasma membrane or during recycling events between this membrane and intracellular organelles.

Whether the more rapid appearance of PrP^{Sc} in the tunicamycin-treated cells is related to the reduced stability of PrP^{C} (Fig. 4) is not known. It has been reported in other systems that asparagine-linked carbohydrates can be crucial for the attainment of proper protein folding and that improper protein conformation can result in decreased stability and enhanced protein aggregation (36, 37). If PrP^{Sc} results from a misfolding of normal PrP polypeptides or from their aggregation (possibly with other molecules), then a putative decreased conformational stability of unglycosylated PrP could conceivably accelerate the appearance of the abnormal PrP^{Sc}. Alternatively, the reduced $t_{1/2}$ for unglycosylated PrP^{Sc} formation could be the indirect consequence of aberrant intracellular localization of its PrP precursor.

We have not addressed the question of the scrapie infectivity associated with the unglycosylated PrP^{Sc}. While the asparagine-linked carbohydrates of PrP^{Sc} are not essential for its synthesis, they may conceivably be of importance in functions related to prion infectivity. For instance, they could mediate the attachment of prions to target cells or feature in distinguishing different scrapie prion isolates (38). The presence of endogenous scrapie infectivity in ScN_2a cells prevented the detection of changes in the infectivity introduced by *de novo* synthesis of unglycosylated PrP^{Sc} in the presence of tunicamycin. It would be interesting to determine whether unglycosylated PrP^{Sc} supports scrapie infectivity by constructing transgenic mice (9) expressing a mutagenized hamster PrP gene lacking the two asparagine-glycosylation sites and determining whether infectious hamster scrapie prions can be propagated in these animals (9, 27).

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