Blockade of Glycosylation Promotes Acquisition of Scrapie-like Properties by the Prion Protein in Cultured Cells*

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The conformational conversion of the prion protein, a sialoglycoprotein containing two N-linked oligosaccharide chains, from its normal form (PrP^C) to a pathogenic form (PrP^Sc) is the central causative event in prion diseases. Although PrP^Sc can be generated in the absence of glycosylation, there is evidence that oligosaccharide chains may modulate the efficiency of the conversion process, and may also serve as molecular markers of diverse prion strains. In addition, mutational inactivation of one of the N-glycosylation sites has recently been associated with a familial spongiform encephalopathy. To investigate the role of N-glycans in determining the properties of PrP, we have expressed in transfected Chinese hamster ovary cells mouse PrP molecules in which N-glycosylation has been blocked either by treatment with the drug tunicamycin, or by substitution of alanine for threonine at one or both of the N-X-T consensus sites. We report that PrP molecules mutated at Thr^182 alone or at both Thr^182 and Thr^196 fail to reach the cell surface after synthesis, but that those mutated at Thr^196 or synthesized in the presence of tunicamycin can be detected on the plasma membrane. We also find that all three mutant PrPs, and to a limited extent wild-type PrP synthesized in the presence of inhibitor, acquire biochemical attributes reminiscent of PrP^Sc. We suggest that the PrP molecule has an intrinsic tendency to acquire some PrP^Sc-like properties, and that N-glycan chains protect against this change. However, pathogenic mutations, or presumably contact with exogenous prions, are necessary to fully convert the protein to a PrP^Sc state.

Prion diseases are fatal neurodegenerative disorders of human beings and animals which have attracted enormous attention among both scientists and the public. The recent emergence of a variant form of Creutzfeldt-Jakob disease in the United Kingdom and France has raised widespread concern among both scientists and the public. The recent emergence of a variant form of Creutzfeldt-Jakob disease in the United Kingdom and France has raised widespread concern among both scientists and the public. This paper is available on line at http://www.jbc.org

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1 The abbreviations used are: GPI, glycosylphosphatidylinositol; CHO, Chinese hamster ovary; endo H, endoglycosidase H; moPrP, mouse prion protein; PAG, polysaccharide gel electrophoresis; PIPCLC, phosphatidylcholin-specific phospholipase C; PrP^C, cellular isoform of the prion protein; PrP^Sc, scrapie isoform of the prion protein.
synthesized in scrapie-infected cells displays a preponderance of lower molecular weight glycoforms (13, 16). This suggests that less glycosylated molecules may be preferred substrates in the generation of PrPSc, an interpretation consistent with studies on the in vitro conversion of unglycosylated PrPC into PrPSc (17). Third, an inherited form of spongiform encephalopathy has recently been described in a Brazilian family with an threonine → alanine substitution at codon 183 which abolishes one of the consensus sites for glycosylation (18). Taken together, these results raise the possibility that the absence of oligosaccharide chains on the PrP molecule may actually increase the efficiency with which PrPSc is formed.

The pattern of PrP glycosylation also appears to be an important marker of prion strain variation. Kacsak et al. (19) noted differences in the immunoblot pattern of PrP species derived from several different rodent scrapie strains. More recently, Parchi et al. (20) have characterized two different PrPSc strains in patients with sporadic Creutzfeldt-Jakob disease that differ in the size and glycosylation pattern of the core fragment (PrP 27–30) produced after protease digestion. Collinge et al. (21) have expanded this classification to include infectious forms of Creutzfeldt-Jakob disease. They report that the PrP 27–30 molecules associated with “new variant” Creutzfeldt-Jakob disease and with bovine spongiform encephalopathy display the same Mₙ and preponderance of the doubly glycosylated isoform, raising the possibility that the BSE agent may have been transmitted to humans. Variations in molecular size and glycoform distribution also characterize PrP 27–30 molecules prepared from the brains of patients with familial prion diseases (22), and we have reported a similar phenomenon in mutant PrP molecules expressed in cultured cells (23). It has been suggested that differences in glycosylation may not only be markers of prion strain variation, but may actually play a causal role in the propagation of strains by targeting each prion variant to distinctive subpopulation of neurons in the brain (24).

Given the likely importance of N-glycosylation to prion biology as suggested by these results, we decided to undertake a detailed analysis of the biosynthesis in cultured cells of PrP molecules that were aberrantly glycosylated, either as a result of mutation of one or both of the glycosylation consensus sites, or because of synthesis in the presence of the inhibitor tunicamycin. One of the mutations we have examined is homologous to the threonine → alanine substitution at codon 183 that is associated with spongiform encephalopathy in the Brazilian family (18). A homologous set of mutations has been examined by Rogers et al. (25) for their effects on trafficking of hamster PrP in CV1 cells. In view of the evidence cited above that absence of glycosylation may improve PrP as a substrate for generation of PrPSc, we were particularly interested in determining whether the abnormally glycosylated molecules would convert spontaneously to a PrPSc-like state. This question was also a natural one to ask, given our previous work in which we showed that PrP molecules carrying other disease-associated mutations acquire all of the biochemical hallmarks of PrPSc when expressed in cultured Chinese hamster ovary (CHO) cells (11, 23, 26, 27). We report here that mutation of either one or both of the consensus sites for N-glycosylation induced PrP to acquire all of the biochemical properties of PrPSc. Surprisingly, even wild-type PrP acquired some of these properties, although to a more limited extent, when glycosylation was inhibited with tunicamycin.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Cell culture reagents were from the Tissue Culture Support Center at Washington University, N-Glycosidase F and tunicamycin from Boehringer Mannheim, sulfo-biotin-NHS from Calbiochem, [35S]methionine (Pro-mix, 1,000 Ci/mmol) from Amer sham, and fluorescein-conjugated secondary antibodies from Cappel. All other reagents were from Sigma. Phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus thuringiensis was prepared as described previously (28).

Mouse monoclonal antibody 3F4 (29) and rabbit polyclonal antibody 78295, raised against PrP 27–30 from scrapie-infected hamster and mouse brain, respectively, were gifts from Dr. Rick Kacsak. Although both of these antibodies react with hamster PrP, CHO cells do not synthesize detectable levels of endogenous hamster PrP, so that only recombinant moPrP is detected in this cell type.

**PrP Constructs**—T182A and T198A mutations were obtained by pol ymerase chain reaction using the following primers: 5’-GACCCA- GAAGGTTATGCGCCACCTTGGCTACTG-3’ (primer 1), 5’-GACCCG- TGTGCTCTGATGCGCGATATTGACCGCTG-3’ (primer 2), 5’- CATCTCCCATACGCGGTCTGGCAGAAAGTTACTCCCCGTTCGTT-3’ (primer 3), 5’-GACAGGAGATCCTCATCACCAGAAGAAG-3’ (primer 4), 5’-CACAGTGGCCCATATCAGCGGCTAAAGCAGACGTCTC-3’ (primer 5), and 5’-ACCAAGGGGGAAGAACCTTCCGCAGACCGATGT- -GAAGATG-3’ (primer 6). The 5’-half of a 3F4-tagged wild-type moPrP cDNA (11) was amplified with primers 1 and 2 (T182A), or with primers 1 and 3 (T198A). The 3’-half of the same cDNA was amplified with primers 4 and 5 (T182A), or with primers 4 and 6 (T198A). 5’ and 3’ polymerase chain reaction products were then mixed, and amplified again with primers 1 and 6 that enable cleavage with HindIII and BamHI. The secondary polymerase chain reaction products were cloned into pBC12/CMV (30) that had been cleaved with the same two enzymes. The T182A/T198A construct was obtained by introducing the T198A mutation into the T182A cDNA. The structure of each DNA construct was confirmed by sequencing.

**Cell Lines**—CHO cells were grown in minimal essential medium containing 7.5% fetal calf serum, and penicillin/streptomycin in an atmosphere of 5% CO₂, 95% air. CHO cells were transfected using Lipofectamine (Life Technologies, Inc.), according to the manufacturer’s directions, with a 1:10 mixture of pR5Vneo (31) and the pBC12/CMV expression plasmid encoding moPrP. Antibiotic-resistant clones were selected in 700 μg/ml genetin (G418), subcloned once, and maintained in 300 μg/ml gentamicin. The experiments reported here were carried out on a single cell line expressing each construct but results were confirmed on additional independent cell lines. The amount of protease-resistant PrP did not change significantly during passage of the cells.

**Metabolic Labeling and Immunoprecipitation**—Confluent cultures of CHO cells were labeled in methionine-free minimal essential medium containing [35S]methionine (Pro-mix; 250–500 μCi/ml), and were chased in Opti-MEM (Life Technologies, Inc.). Cells were then lysed in a buffer that contained 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5 mM Triton X-100, and 0.5 mM sodium deoxycholate, supplemented with protease inhibitors (peptatin and leupeptin, 1 μg/ml; phenylmethylsulfonyl fluoride, 0.5 mM; EDTA, 2 mM). Lysates were first clarified by centrifugation for 5 min at 16,000 × g in a microcentrifuge, a procedure that removes debris but does not pellet significant amounts of PrP. Immunoprecipitation of PrP from clarified lysates was performed using monoclonal antibody 3F4 as described previously (11). Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Deglycosylation of PrP**—Where indicated, cell lysates were treated prior to immunoprecipitation with 0.01 units/ml N-glycosidase F for 16 h at 37 °C. For the experiments shown in Fig. 3, immunoprecipitated PrP was eluted from protein A-Sepharose with 20 μl of 50 mM sodium citrate (pH 5.5) containing 0.1% SDS, and then incubated for 16 h with either endoglycosidase H (1.6 units/μl) or neuraminidase (10 units/μl). After addition of 2 × SDS sample buffer, samples were analyzed by SDS-PAGE.

**Immunocytochemistry**—Cells grown on glass coverslips were incubated with polyclonal antibody 78295 for 1 h at 4 °C. After rinsing, cells were fixed with methanol for 30 min at 4 °C, and then incubated with fluorescein-conjugated secondary antibody for 1 h at room temperature. Coverslips were mounted in glycerol, and cells observed with a Nikon Optiphot-2 fluorescence microscope.

**Assay of Trypsin Accessibility**—Metabolically labeled cells were rinsed with phosphate-buffered saline, and were either left untreated, or were incubated for 6 min at 4 °C with 0.25% trypsin in phosphate-buffered saline followed by addition of fetal calf serum to 10% to terminate digestion. MoPrP was then immunoprecipitated from cell lysates with 3F4 antibody as described above.

S. Lehmann and D. A. Harris, unpublished data.
**RESULTS**

**Expression of Wild-type, T182A, T198A, and T182A/T198A moPrPs in Transfected CHO Cells**—We have previously used CHO cells to express recombinant mouse PrP (moPrP), and have shown that in these cells PrP molecules carrying pathogenic mutations are converted to a PrPSc-like state (11, 23, 26, 27). For the present studies, we have analyzed stably transfected CHO cells that express wild-type moPrP, as well as moPrP molecules carrying mutations in the consensus sites for N-glycosylation. Like other mammalian PrPs, moPrP contains two consensus sites for asparagine-linked glycosylation (N-X-T, and N-F-T, spanning residues 180–182 and 196–198, respectively). We constructed moPrP molecules containing either one or both of the following point mutations: T182A and T198A (Fig. 1). These mutations, by changing the third amino acid of the consensus sequence N-X-T, abolish utilization of the corresponding glycosylation site. All moPrP constructs were tagged with an epitope for the monoclonal antibody 3F4 to facilitate their detection in immunoblotting and immunoprecipitation experiments.

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**Fig. 1. Schematic of moPrP glycosylation mutants.** Numbers indicate amino acid positions. N-T and N-F-T are consensus sites for N-linked glycosylation. Alanine was substituted for threonine in each site singly (T182A and T198A) and in both sites together (T182A/T198A). Substitution of methionine residues at positions 108 and 111 creates an epitope for the monoclonal antibody 3F4.

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**Fig. 2 (lane 1) shows that wild-type moPrP expressed in transfected CHO cells appeared as three major bands at 28, 30–32, and 35–45 kDa. The 28-kDa band represents unglycosylated molecules, the 30–32-kDa band molecules with a single N-linked oligosaccharide chain (attached to either Asn180 or Asn196), and the 35–45-kDa band molecules with two oligosaccharide chains (attached to both Asn180 and Asn196). The singly and doubly glycosylated species migrate as broad bands on SDS-PAGE, presumably due to heterogeneous modification of their core oligosaccharides. Since the cells in this experiment had been labeled for 2.5 h and chased for 30 min, the bands represent the mature glycoforms. A similar pattern of glycosylation of wild-type PrP has been described in other cell types by a number of authors (10, 12, 13).

The mutant moPrPs each displayed a distinctive pattern of glycosylation. T182A moPrP (Fig. 2, lane 3) appeared as a sharp band of 31 kDa representing the singly glycosylated species. The T198A protein (lane 5) migrated as a major band of 31 kDa corresponding to singly glycosylated molecules, and a minor band of 28 kDa corresponding to unglycosylated protein. The 31-kDa band was usually broad in appearance, with a faint smear extending above it, suggesting that this species, like the corresponding glycoform of wild-type moPrP, carried a single oligosaccharide chain with heterogeneous modifications of its core glycans. When both sites were mutated (T182A/T198A), the protein migrated at 28 kDa, although it was sometimes possible to detect a minor band of ~30 kDa (see lane 7 of Fig. 6B) which may reflect glycosylation of a non-canonical site (25).

Deglycosylation with N-glycosidase F resolved wild-type and mutant moPrPs into a single species of 28 kDa (Fig. 2, lanes 2, 4, 6, and 8), corroborating the assumption that variations in molecular mass of the PrP species results from addition of N-linked glycan chains. Also consistent with this idea, wild-type PrP synthesized in the presence of tunicamycin migrated as a single PrP band of 28 kDa (lane 9). Tunicamycin inhibits the addition of GlcNAc to dolichol phosphate, the first step in construction of the core oligosaccharide structure that is transferred to the asparagine residue of polypeptides to be glycosylated.

T198A moPrP, but Not T182A moPrP, Is Susceptible to Digestion by Endoglycosidase H and Is Resistant to the Action of Neuraminidase—To follow maturation of the oligosaccharide chains of the recombinant moPrPs, we tested their susceptibility to digestion by endoglycosidase H (endo H) and neuraminidase (Fig. 3). Complex N-linked oligosaccharides become resistant to the action of endo H in the mid-Golgi as a result of the activity of the enzyme mannosidase II, and if they contain sialic acid residues they become susceptible to attack by neuraminidase. T198A moPrP, but Not T182A moPrP, is resistant to the action of neuraminidase, whereas T182A moPrP is susceptible.
dase in the trans-Golgi network where the enzyme sialyl transferase resides. When cells were pulse-labeled for 90 min and chased for 30 min, wild-type moPrP was resistant to digestion by endo H, and underwent a small but consistent increase in its electrophoretic mobility after digestion with neuraminidase (lanes 1–3). This result is consistent with our previous demonstration that wild-type moPrP in CHO cells transits to the cell surface within 30 min after synthesis (11). In contrast to the wild-type protein, T182A moPrP was endo H-sensitive and neuraminidase-resistant under the same conditions (lanes 4–6), suggesting the mutant polypeptide failed to reach the mid-Golgi. Surprisingly, T198A moPrP was endo H-resistant and neuraminidase-sensitive (lanes 6–9), indicating that it transited as far as the trans-Golgi network; because of the small size of the mobility shift induced by neuraminidase, it was not possible to estimate whether all or only a fraction of the molecules became sialylated. The broadness of the 31-kDa band is also consistent with the acquisition of terminal sugar modifications on at least some of the molecules. As expected, cells expressing wild-type moPrP displayed intense plasma membrane staining (Fig. 4A). In contrast, cells expressing T182A moPrP were stained only at background levels (Fig. 4B), consistent with our observation that this mutant protein fails to become endo H-resistant, and suggesting that it remains trapped intracellularly in a compartment proximal to the mid-Golgi. The double mutant T182A/T198A also apparently fails to reach the cell surface, as judged by lack of surface staining (Fig. 4D). Cells expressing T198A moPrP displayed surface fluorescence that was distinctly higher than for cells expressing the other mutants (Fig. 4C), consistent with the idea that at least some of the T198A molecules were transported to the plasma membrane following sialylation in the trans-Golgi network. The fact that the staining intensity was less than that of cells expressing wild-type moPrP, however, indicates that surface transport of T198A is inefficient.

To confirm these immunocytochemical observations, we analyzed surface expression of the moPrPs by assaying their accessibility to digestion by externally applied trypsin following metabolic labeling (Fig. 5, lanes 1–8). We found that after 4 h of continuous labeling, 59% of wild-type moPrP and 34% of T198A moPrP were susceptible to digestion by trypsin, compared with ~5% for T182A and T182A/T198A moPrPs (Table I). Additional evidence that T198A moPrP was present on the cell surface is the fact that it can be labeled by biotinylation of intact cells with the membrane-impermeant reagent sulfo-biotin-X-NHS (Fig. 6C, lane 6).

Mutant moPrPs Acquire PrPSc-like Properties—PrPSc can be
distinguished from PrP<sup>C</sup> based on several biochemical properties, including: 1) insolubility in non-denaturing detergents (33); 2) relative resistance to digestion by proteinase K, which produces a core fragment of 27–30 kDa (33); 3) failure to be released from the cell surface by treatment with the GPI anchor-cleaving enzyme PIPLC (23, 34); and 4) retention in the detergent phase after PIPLC digestion and Triton X-114 phase partitioning (11, 27). We have shown previously that PrP molecules carrying disease-related mutations acquire all four of these properties when expressed in CHO cells (11, 23, 26, 27).

We have hypothesized that the last two properties are related to the existence of a secondary mechanism of membrane attachment in addition to the GPI anchor, and to a conformational alteration of the protein that renders the GPI anchor partially inaccessible to PIPLC (11, 27).

We tested whether the three moPrP glycosylation mutants acquired each of these PrP<sup>Sc</sup>-like properties. We assessed detergent-insolubility by centrifuging lysates of metabolically labeled CHO cells at 266,000 × g for 40 min, a protocol that sediments PrP<sup>Sc</sup> but not PrP<sup>C</sup>. Under these conditions, 35–50% of T182A, T198A, and T182A/T198A moPrPs sedimented, compared with 3% for wild-type moPrP (Fig. 6A and Table I).

To test protease resistance, proteins methanol-precipitated from cell lysates were treated for 10 min with 3.3 μg/ml proteinase K (Fig. 6B). We observed that T182A, T198A, and T182A/T198A moPrPs were cleaved by the protease to yield a fragment that migrated around 24 kDa (lanes 4, 6, and 8), while under the same conditions wild-type moPrP was completely degraded (lane 2). The fragment produced by digestion of the three glycosylation mutants has a smaller molecular mass than PrP<sup>27–30</sup>, the protease-resistant core of PrP<sup>Sc</sup> from brain, presumably because the proteins from which they are derived are underglycosylated. Indeed, the fact that all three mutants produced a fragment of similar size suggests that the protease-resistant core was derived primarily from the unglycosylated form of each protein, a conclusion that is confirmed by the lack of effect of tunicamycin on the mobility of the fragments (data not shown).

Because T198A is the only one of the three mutant PrPs to be expressed on the cell surface (see above), this is the only one that could be tested for its susceptibility to release by PIPLC. We found that T198A molecules labeled by surface biotinylation were almost completely retained on the cell surface following treatment with PIPLC (Fig. 6C, lanes 5 and 6), in contrast to wild-type molecules, the majority of which were released into the medium (Fig. 6C, lanes 1 and 2) (Table I). Interestingly, the small amount of unglycosylated wild-type moPrP that was detectable on the cell surface in some experiments was not released by PIPLC (Fig. 6C, lane 2). As expected from their inaccessibility to surface staining and trypsin digestion, both Thr<sup>182</sup> and T182A/T198A moPrPs were not labeled by surface biotinylation (Fig. 6C, lanes 3, 4, 7, and 8).

When we treated Triton X-114 lysates of metabolically labeled cells with PIPLC and then partitioned them at 37 °C, we

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3 S. Lehmann and D. A. Harris, unpublished observations.
the presence of tunicamycin displayed to a limited extent some, but not all, of the biochemical properties of PrPSc. Approximately 15% of the protein from the inhibitor-treated cells was found in the pellet after ultracentrifugation, and a similar proportion partitioned into the Triton X-114 phase after PI-PLC treatment, values that are significantly different from those for untreated cells (Fig. 7, A and D; Table I). Virtually all of the moPrP synthesized in the presence of tunicamycin was retained on the cell surface after treatment with PIPLC (Fig. 7C; Table I). Thus, the protein synthesized in the presence of tunicamycin displays three of the biochemical properties of PrPSc, although the degree of detergent-insolubility and hydrophobicity in the Triton X-114 assay are less than for the three mutant moPrPs (Table I). When we tested the proteinase K sensitivity of moPrP molecules synthesized in the presence of tunicamycin, we were unable to detect a protease-resistant fragment (Fig. 7B, lane 4).

To confirm these results in another cell type, we performed

### Table I

Properties of moPrPs

<table>
<thead>
<tr>
<th>Detergent insolubility (% in pellet)</th>
<th>Protease resistance (% remaining)</th>
<th>Surface retention (% on cells after PIPLC)</th>
<th>Triton X-114 partitioning (% in detergent phase)</th>
<th>Trypsin accessibility (% digested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2.7 ± 0.6</td>
<td>0.6 ± 0.4</td>
<td>14.9 ± 4.4</td>
<td>7.6 ± 3.1</td>
</tr>
<tr>
<td>Wild-type + tunicamycin</td>
<td>13.3 ± 2.8</td>
<td>1.1 ± 0.7</td>
<td>95.3 ± 4.0</td>
<td>15.6 ± 2.8</td>
</tr>
<tr>
<td>T182A</td>
<td>36.3 ± 4.5</td>
<td>10.9 ± 2.2</td>
<td>96.8 ± 2.4</td>
<td>62.0 ± 8.3</td>
</tr>
<tr>
<td>T198A</td>
<td>48.4 ± 4.3</td>
<td>18.8 ± 3.5</td>
<td>60.1 ± 6.3</td>
<td>33.9 ± 3.9</td>
</tr>
<tr>
<td>T182A/T198A</td>
<td>42.7 ± 3.3</td>
<td>11.6 ± 2.4</td>
<td>67.4 ± 10.4</td>
<td>4.9 ± 2.0</td>
</tr>
<tr>
<td>T182A: T198A</td>
<td>42.7 ± 3.3</td>
<td>11.6 ± 2.4</td>
<td>67.4 ± 10.4</td>
<td>4.9 ± 2.0</td>
</tr>
<tr>
<td>Wild-type tunicamycin</td>
<td>13.3 ± 2.8</td>
<td>NA</td>
<td>62.0 ± 8.3</td>
<td>5.6 ± 1.6</td>
</tr>
<tr>
<td>T182A</td>
<td>36.3 ± 4.5</td>
<td>NA</td>
<td>62.0 ± 8.3</td>
<td>5.6 ± 1.6</td>
</tr>
<tr>
<td>T198A</td>
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<td>62.0 ± 8.3</td>
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<tr>
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<td>42.7 ± 3.3</td>
<td>NA</td>
<td>62.0 ± 8.3</td>
<td>5.6 ± 1.6</td>
</tr>
</tbody>
</table>

*NA, not applicable.

**Fig. 6.** T182A, T198A, and T182A/T198A moPrPs acquire biochemical properties PrPSc. Panel A, lysates of CHO cells metabolically labeled for 3 h were centrifuged at 265,000×g to separate detergent-soluble from detergent-insoluble molecules. MoPrP in supernatant (S) and pellet (P) fractions was immunoprecipitated and separated by SDS-PAGE. Prior to immunoprecipitation, samples were enzymatically deglycosylated to produce a single band of PrP that could be more easily quantitated. Panel B, proteins methanol-precipitated from detergent lysates of CHO cells were left undigested (− lanes), or were treated with proteinase K (3.3 μg/ml) at 37 °C for 10 min (+ lanes). Proteins were then separated by SDS-PAGE and immunoblotted using antibody 3F4. Four times as many cell equivalents were loaded in the + lanes as in the − lanes. Panel C, cells were surface-biotinylated and then treated with PIPLC at 4 °C. MoPrP in PIPLC incubation media (M) and cell lysates (C) was immunoprecipitated, separated by SDS-PAGE, and then visualized by developing blots of the gels using horseradish peroxidase-coupled streptavidin and enhanced chemiluminescence. Panel D, lysates of CHO cells metabolically labeled for 20 min were subjected to Triton X-114 phase partitioning. MoPrP in aqueous (A) and detergent (D) phases was immunoprecipitated after enzymatic deglycosylation and separated by SDS-PAGE.
the same experiments on endogenous moPrP synthesized by untransfected mouse N2a neuroblastoma cells that had been treated with tunicamycin. We found that this protein behaved similarly to the one from inhibitor-treated CHO cells, in terms of its detergent-insolubility, PIPLC-releasability, and hydrophobicity in the Triton X-114 assay (results not shown).

DISCUSSION

N-Linked glycosylation of PrP is likely to play a critical role in prion biology, based on recent evidence that this modification may be a marker of prion strain diversity (20–22), and that an inherited form of spongiform encephalopathy is associated with mutational inactivation of one of the two consensus sites for N-glycosylation (18). To explore the role of N-glycosylation in PrP metabolism, we have carried out a detailed analysis of transfected CHO cells that express abnormally glycosylated forms of PrP, generated either by substitution of alanine for threonine in one or both of the N-X-T consensus sites, or by synthesis in the presence of tunicamycin. We report here that PrPs carrying glycosylation site mutations display all of the biochemical properties of PrPSc, and that wild-type PrP synthesized in the presence of tunicamycin exhibits a subset of these properties to a limited extent. Our results suggest that the PrP molecule has an intrinsic tendency, promoted by the absence of N-linked glycans, to become detergent-insoluble, PIPLC non-releasable, and hydrophobic, but that these properties do not become prominent unless the protein is converted to the PrPSc state as a result of mutation.

Biosynthesis and Trafficking of Abnormally Glycosylated PrP—We first characterized the metabolism and localization of PrP molecules lacking one or both N-linked oligosaccharide chains. Our results suggest that the two glycosylation consensus sites are not equivalent in terms of the effect that mutagenesis of these sites has on the trafficking of PrP. Inactivation of the site surrounding asparagine 180 (in T182A moPrP) blocked delivery of the protein to the cell surface. The intracellular compartment where this protein resides remains to be determined, but it is likely to be proximal to the mid-Golgi stack since the mutant protein remained sensitive to endo H. The double mutant T182A/T198A moPrP was also absent from the cell surface, and it is possible that its transit is blocked at the same point as T182A moPrP.

In contrast, mutation of the site surrounding asparagine 196 (in T198A moPrP) reduced, but did not completely prevent, delivery of the protein to the plasma membrane. Based on the relative surface staining intensity of cells expressing T198A moPrP compared with cells expressing equivalent amounts of wild-type moPrP (Fig. 4), and on the fact that only about 30% of the T198A protein was susceptible to trypsin digestion compared with about 60% for the wild-type protein (Table I), we estimate that only about half of the mutant molecules reach the cell surface after synthesis. The localization of those molecules that fail to reach the plasma membrane remains to be determined, but it is likely that they reside in a compartment distal to the mid-Golgi, based on the fact that virtually all of the mutant molecules became endo H-resistant.

Treatment of cells with tunicamycin did not appreciably affect delivery of wild-type moPrP to the cell surface. This indicates that N-linked glycans are not necessary for normal biosynthetic trafficking of PrP. This conclusion, which was also reached by Petersen et al. (12), is consistent with the observa-
Glycosylation of the Prion Protein

Our results on the biosynthesis and trafficking of moPrP glycosylation mutants in CHO cells are similar in most respects to those of Rogers et al. (25) who analyzed a homologous set of hamster PrP mutants in CV1 cells. In contrast to us, however, they failed to detect any of the mutant proteins on the cell surface, and they speculated that the absence of oligosaccharide chains per se was responsible for this effect. This contention seems unlikely in light of our demonstration that T198A moPrP as well as wild-type moPrP in the presence of tunicamycin both reach the cell surface.

Scrapie-like Properties of Aberrantly Glycosylated PrP—We found that T182A, T198A, and T182A/T198A moPrPs each exhibited three of the four operational properties that we have used previously to define the PrPSc state: 1) insolubility in Triton/deglycosylate; 2) production of a protease-resistant core fragment after digestion with proteinase K; and 3) retention in the Triton X-114 detergent phase after PIPLC treatment. T198A moPrP was the only mutant that could be assayed for the fourth property, retention on the cell surface after PIPLC treatment, since this was the only one of the proteins that was expressed on the plasma membrane, and it was found to be PIPLC non-releasable. The T182A mutation is homologous to one in human PrP that has recently been found in a Brazilian family with an inherited spongiform encephalopathy (18). The fact that a moPrP molecule carrying this mutation acquires PrPSc-like properties is therefore consistent with our previous work showing that six other disease-related mutations, but not a non-pathogenic amino acid substitution, induced a PrPSc-like state when expressed in cultured CHO cells (23, 35). We do not yet know whether mutant PrPs synthesized in CHO cells are infectious. Although no individuals carrying a mutation homologous to T198A have yet been described, we would predict that such individuals might be found in the future.

Our results have implications for the cellular location at which PrPSc is generated. The fact that T182A and T182A/T198A moPrPs are converted to a PrPSc-like state without being expressed on the cell surface indicates that transit to the plasma membrane is not necessary for the conversion process. This conclusion is consistent with our recent studies of another mutant PrP which indicate that the earliest step in generation of PrPSc occurs in the endoplasmic reticulum during or very soon after synthesis of the polypeptide chain, and is manifested by retention of the protein in the Triton X-114 phase after PIPLC treatment (27). This change is followed later by acquisition of detergent insolubility and protease resistance, steps that normally take place subsequent to arrival of the newly synthesized protein at the plasma membrane, but which can occur intracellularly in cells treated with brefeldin A. The conclusion that emerges from our studies, as well as ones using scrapie-infected neuroblastoma cells (15), is that PrPSc production can occur in any of several cellular compartments, depending on the protein being expressed and the presence of pharmacological agents. It is noteworthy that none of six other disease-related mutations that we have analyzed previously markedly altered the efficiency with which PrP is delivered to the cell surface (11, 23, 35). These mutations include five single amino acid substitutions and one insertion, covering both N-terminal and C-terminal halves of the molecule. This comparison suggests that defects in biosynthetic trafficking are not a general feature of pathologic mutations in PrP, and are not necessary for generation of PrPSc. For the T198A mutant, which exhibits only a partial block in cell surface delivery, it may be the retained or non-retained molecules, or both, that are converted to the PrPSc state.

Surprisingly, wild-type PrP synthesized in the presence of tunicamycin displayed several biochemical attributes of PrPSc, although less prominently than the mutant PrPs. We were unable to detect any protease-resistant PrP in tunicamycin-treated cells, although ~5% of the protein was detergent-insoluble after ultracentrifugation and hydrophobic in the Triton X-114 assay, and most of it was retained on the cell surface after treatment with PIPLC. These results raise the question of whether some of the PrP synthesized in the presence of tunicamycin might have undergone the conformational transition to authentic PrPSc. We think that this is unlikely for at least two reasons. First, unglycosylated wild-type PrP constitutes 5–10% of the PrP synthesized in cells normally, and it would be surprising if these molecules represented PrPSc. This small population of unglycosylated PrP in control cells was poorly released by PIPLC (Fig. 6C, lanes 1 and 2), suggesting that it is similar to PrP synthesized in tunicamycin-treated cells. We have not tested these molecules for other PrPSc-like properties. Second, wild-type PrP molecules from tunicamycin-treated cells are clearly less “scrapie-like” than mutant PrP molecules. All seven mutant PrPs associated with familial spongiform encephalopathies that we have analyzed in CHO cells thus far (six published previously (23, 35) and T182A presented here) produce protease-resistant fragments, and they all display a degree of detergent-insolubility and hydrophobicity in Triton X-114 that are greater than those of wild-type PrP synthesized in the presence of tunicamycin. T182A/T198A moPrP, which carries no N-linked glycans, is also distinct in its trafficking and biochemical properties from unglycosylated wild-type protein made in inhibitor-treated cells. We conclude from these comparisons that alteration of the amino acid sequence changes the state of PrP in a way that inhibition of glycosylation alone does not. We have noted previously that some PrP molecules carrying pathogenic mutations display an altered glycoform distribution, with a shift toward less glycosylated forms (23, 35). In light of the discussion here, we would suggest that the PrPSc-like properties of these molecules are determined directly by mutation-induced alterations in protein conformation rather than indirectly via a change in glycosylation state.

There are several possible explanations for why PrP from tunicamycin-treated cells displays limited scrapie-like properties. First, it is conceivable that some of the PrP synthesized in these cells has been converted to a state that is intermediate between PrPSc and PrPSc. It has been proposed that even normal cells contain small amounts of a hypothetical species denoted PrP* that is an obligatory intermediate in the transformation of PrPC to PrPSc (8); other authors have suggested the existence of related intermediate species (36, 37). One might expect these species to possess some but not all of the biochemical markers of PrPSc or display these markers to a quantitatively more limited extent. The amount of this intermediate may be undetectable in normal cells, but its amount might be increased in tunicamycin-treated cells because the absence of N-linked glycans favors the transition from PrPC to PrP*. A second possible explanation is that the PrP molecule has an intrinsic propensity to adopt certain of the biochemical attributes of PrPSc even when it is in the PrPC state, and that glycosylation tends to prevent this. In this view, the biochemical markers we and others commonly use to recognize PrPSc
are not unique to that isoform, although conversion to the PrPSc state significantly accentuates these operational properties. A third possibility is that the insolubility and PIPLC resistance we observe in unglycosylated PrP are not directly related to the formation of PrPSc, and that they simply reflect changes in physical properties that might occur in any glycoprotein synthesized without its normal complement of oligosaccharide chains. It is well known, for example, that the polarity of N-glycans enhances the solubility of proteins to which they are attached, and that their absence can promote aggregation (14).

Regardless of which of these explanations should prove to be correct, our results are consistent with several pieces of evidence suggesting that unglycosylated molecules are somehow preferred, either as substrates or products, in the formation of PrPSc. PrPSc is produced more rapidly in scrapie-infected neuroblastoma cells treated with tunicamycin (15), and PrPSc synthesized PrPSc, and use of additional glycosylation inhibitors and pathway, enzymatic modification of the glycan chains of purified PrPSc, and use of additional glycosylation inhibitors and removal or modification of glycan chains, and targeting of selective degradation of specific glycoforms (12), enzymatic changes in physical properties that might occur in any glyco-

REFERENCES


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Additions and Corrections


Human N-myristoyltransferase amino-terminal domain involved in targeting the enzyme to the ribosomal subcellular fraction.

Constance J. Glover, Kathleen D. Hartman, and Ronald L. Felsted

Page 28685, Table II: Bases 6–49 were found to correspond to the sequence of the CLONTECH Marathon cDNA Adaptor used in our 5'-RACE analysis. This correction eliminates the two identified stop sites and restores the possibility that hNMT may be translated from a start site even further upstream from that proposed in this study.

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Blockade of glycosylation promotes acquisition of scrapie-like properties by the prion protein in cultured cells.

Sylvain Lehmann and David A. Harris

Page 21479, line 18 of the abstract should read:

We report that PrP molecules mutated at Thr\textsuperscript{182} alone or at both Thr\textsuperscript{182} and Thr\textsuperscript{198} fail to reach the cell surface after synthesis, but that those mutated at Thr\textsuperscript{198} or synthesized in the presence of tunicamycin can be detected on the plasma membrane.


Kinetic mechanism of GTP binding and RNA synthesis during transcription initiation by T7 RNA polymerase.

Yiping Jia and Smita S. Patel

Page 30148, Scheme 1: The scheme was inadvertently cut in half. The complete scheme is shown below:

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\text{SCHEME 1}
\]

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Blockade of Glycosylation Promotes Acquisition of Scrapie-like Properties by the Prion Protein in Cultured Cells
Sylvain Lehmann and David A. Harris

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