Prion Propagation in Cells Expressing PrP Glycosylation Mutants

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Infection by prions involves conversion of a host-encoded cell surface protein (PrP<sup>C</sup>) to a disease-related isoform (PrP<sup>Sc</sup>). PrP<sup>C</sup> carries two glycosylation sites variably occupied by complex N-glycans, which have been suggested by previous studies to influence the susceptibility to these diseases and to determine characteristics of prion strains. We used the Rov cell system, which is susceptible to sheep prions, to generate a series of PrP<sup>C</sup> glycosylation mutants with mutations at one or both attachment sites. We examined their subcellular trafficking and ability to convert into PrP<sup>Sc</sup> and to sustain stable prion propagation in the absence of wild-type PrP. The susceptibility to infection of mutants monoglycosylated at either site differed dramatically depending on the amino acid substitution. Aglycosylated double mutants showed overaccumulation in the Golgi compartment and failed to be infected. Introduction of an ectopic glycosylation site near the N terminus fully restored cell surface expression of PrP but not convertibility into PrP<sup>Sc</sup>, while PrP<sup>C</sup> with three glycosylation sites conferred cell permissiveness to infection similarly to the wild type. In contrast, predominantly aglycosylated molecules with nonmutated N-glycosylation sequons, produced in cells expressing glycosylphosphatidylinositol-anchorless PrP<sup>C</sup>, were able to form infectious PrP<sup>Sc</sup>. Together our findings suggest that glycosylation is important for efficient trafficking of anchored PrP to the cell surface and sustained prion propagation. However, properly trafficked glycosylation mutants were not necessarily prone to conversion, thus making it difficult in such studies to discern whether the amino acid changes or glycan chain removal most influences the permissiveness to prion infection.

Transmissible spongiform encephalopathies (TSE), or prion diseases, are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease and related diseases in humans, scrapie in sheep, bovine spongiform encephalopathy in cattle, and chronic wasting disease in cervids. The prion, the transmissible agent, is thought to be made essentially of PrP<sup>Sc</sup>, a misfolded form of the host protein PrP<sup>C</sup>. PrP<sup>Sc</sup> displays a novel conformation enriched in β-sheets, conferring increased protease resistance and a tendency to form amyloid-like multimers. Prion propagation is believed to occur by recruitment and conversion of new PrP molecules within PrP<sup>Sc</sup> seeds. The three-dimensional structure of PrP<sup>Sc</sup> multimers and the conformational dynamic changes underlying PrP<sup>C</sup> conversion remain unresolved (11, 48).

PrP<sup>C</sup> is a glycoprotein that is localized predominantly to detergent-resistant membrane microdomains via a glycosylphosphatidylinositol (GPI) anchor. Two sites for Asn-linked glycosylation are present in the structured, C-terminal half of the protein, both of which are variably occupied, thus producing di-, mono-, and unglycosylated molecules (20). PrP<sup>C</sup> is exposed at the cell surface, from which it is subjected to endocytosis and recycled between the endocytic compartment and the plasma membrane (25). While several roles have been proposed, the biological function of PrP<sup>C</sup> remains elusive (1).

In infected cells, PrP<sup>Sc</sup> accumulates at the cell surface and chronic wasting disease in cervids. The prion, the transmissible agent, is thought to be made essentially of PrP<sup>Sc</sup>, a misfolded form of the host protein PrP<sup>C</sup>. PrP<sup>Sc</sup> displays a novel conformation enriched in β-sheets, conferring increased protease resistance and a tendency to form amyloid-like multimers. Prion propagation is believed to occur by recruitment and conversion of new PrP molecules within PrP<sup>Sc</sup> seeds. The three-dimensional structure of PrP<sup>Sc</sup> multimers and the conformational dynamic changes underlying PrP<sup>C</sup> conversion remain unresolved (11, 48).

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In infected cells, PrP<sup>Sc</sup> accumulates at the cell surface and chronically in intracellularly, mainly in the endolysosomal compartment, but the subcellular site(s) where conversion takes place remains a subject of debate (11, 35).

Natural and rodent-adapted prion strains can be differentiated based on the neuropathological changes they induce in a defined host and the biochemical features of the PrP<sup>Sc</sup> accumulating in the infected tissues (4). Thus, while all PrP glycoforms can acquire protease resistance, the ratio of bi-, mono-, and unglycosylated resistant PrP<sup>Sc</sup> species (PrPres) stably differs among prion strains, a feature commonly used for their molecular typing (27). Whether and how the PrP glycan chains influence the strain specificity of prion formation remain to be clarified.

Cell culture experiments have unambiguously established that not only the strain but also the cells in which the prion replication takes place can determine the PrPres glycoform (5, 43, 59). Moreover, PrP<sup>Sc</sup> glycosylation may differ in individual cell types or brain regions (3, 21). The glycoform ratio associated with a defined strain also varies depending on the brain region or organ in which PrP<sup>Sc</sup> is formed (16, 32, 50, 53). Such observations have led to the speculation that the glycoform ratio of PrP<sup>C</sup> expressed by neuronal cells might be a determinant of the different brain-targeting selectivity manifested by prion strains (15).

Acellular amplification of prions is possible through protein misfolding cyclic amplification (PMCA) (10). In one study where reconstituted PrP<sup>C</sup> material was used as a substrate, the proportion of each glycoform was shown to affect the efficiency of PrP<sup>Sc</sup> formation, therefore arguing that the stoichiometry of the various glycoforms within PrP<sup>Sc</sup> multimers obeys strain-specific conformational constraints (39). Also, inoculation of aglycosylated PrP<sup>Sc</sup> material produced by PMCA into mice provided evidence that the carbohydrate moiety of the input PrP<sup>Sc</sup> is not required for the maintenance of the strain-specific neurotropism (45). Such observations support the view that PrP<sup>Sc</sup> glycoform variation may be a consequence rather than a
cause of the prion strain diversity, with the strain specificity being essentially encrypted by distinct conformations of PrPSc backbone (29, 48).

To what extent host PrP glycosylation influences the outcome of prion infection and disease in a host is an important issue. Studies in transgenic mouse models expressing PrP with no glycans at either one or both sites established that the diglycosylated form of PrP is not mandatory for prion infection (16, 38). More recent work using gene-targeted transgenic mice expressing glycosylation mutants under the control of the endogenous promoter demonstrated that PrP<sup>Sc</sup> glycosylation is not essential for establishing infection within a host by intracerebral inoculation; however, prion replication in mice expressing aglycosylated PrP was fully or strongly impaired, depending on the strain (9, 56). Glycosylation was shown to influence the incubation period before disease onset and not the neuropathological features in the brain (56). Following inoculation by a peripheral route, however, PrP<sup>Sc</sup> glycosylation profoundly influenced both the disease timing and the PrP<sup>Sc</sup> deposition pattern, and mice without PrP glycosylation were resistant (8). This suggested a role for PrP-associated glycans in the spread of prions within the organism. One general limitation of such studies, however, is the limited number of mutant PrPs expressed in mice and hence the possibility that the observed differential strain requirement is linked to the replaced amino acid rather than to the removed glycans. Moreover, there is evidence from experiments both in cultured cells (30, 31, 49) and in vivo (9, 16) that glycosylation-abolishing mutations can alter the intracellular PrP trafficking, thus possibly affecting the efficiency of the conversion process.

The strategy generally employed to address how the glycosylation status of PrP influences its ability to sustain prion propagation has consisted of transiently expressing tagged mouse PrP constructs with mutated glycosylation sites in steadily infected ScN2a mouse cells and monitoring acute conversion into PrP<sup>Sc</sup> (28, 30, 38). In the present study, we have taken advantage of the Rov cell model, which is infectible by ovine prions (58), to further address this issue. The approach used here differs in several aspects. First, the trafficking properties of mutants and their conversion competence were analyzed in cultures that stably expressed only the glycosylation mutant protein, as the concomitant expression of wild-type molecules within the same cells may create a less than optimal context to do this. Second, the conversion competence was examined in a situation of de novo infection and along subpassages of the cultures, which the robustness of the Rov cell infection system made feasible. We observed dramatic differences depending on the amino acid change introduced and that proper trafficking to the cell surface did not suffice to confer permissiveness to infection. These results are important in the perspective of elucidating the specific roles of PrP Asn-glycan chains in prion biology.

**MATERIALS AND METHODS**

**Cell culture.** Rov cells are derived from the epithelial RK13 cell line and express ovine PrP in a doxycycline-inducible manner (58). They were grown in Opti-MEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) and antibiotics and were split 1:4 after trypsin dissociation once a week.

**Generation of PrP mutants.** Mutations were introduced by site-directed mutagenesis (QuikChange II mutagenesis kit; Stratagene) into sheep PrP (allotype V<sup>3078</sup>L<sup>3078</sup>S<sup>3078</sup>), cloned into plasmid pTRE (Clontech) using mutagenic primers.

**Prion infection of cell cultures.** Cells were infected with 127S strain of sheep scrapie as previously described using 1% (wt/vol) of brain pool homogenates of terminally ill g638 mice (58). Two days after exposure, cells were carefully rinsed and incubated for two additional days before trypsinization and passage of cells at a 1:10 dilution (passage 1). Cells were further incubated for 1 week and then split at a 1:4 dilution at each following passage. Rov cells with PrP<sup>Sc</sup> were similarly inoculated with the brain homogenate, incubated for 2 days, and then rinsed and covered with culture medium plus 0.6% agarose. After 1 week of incubation, the agarose layer was mechanically removed using a scalpel blade and replaced by a fresh one, and the cells were further incubated for 1 week prior to harvesting for analysis. The amount of PrPres associated with the removed agarose layer was found to be minimal. To test the infectivity of cultures that presumably propagated mutant N200D PrP<sup>Sc</sup>, 5 × 10<sup>6</sup> cells were harvested at passage 6 postexposure, pelleted, frozen and thawed, and then sonicated and used as inoculum for as brain homogenate. The infectivity of AGPI PrP<sup>Sc</sup> was assessed using material prepared from 2-week-old cultures under agarose by disruption in lysis buffer (see the next section) and methanol precipitation. Resuspended dry pellets were used to inoculate Rov9 cells expressing wild-type PrP (58).

**Cell lysis and PK digestion.** The cells were washed twice with cold phosphate-buffered saline (PBS), and whole-cell lysates were prepared at 4°C in TLI buffer (50 mM Tris-HCl [pH 7.4], 0.5% sodium deoxycholate, 0.5% Triton X-100). Lysates were clarified by centrifugation at 1,000 × g for 2 min, and the protein concentration was determined by bichinchoninic acid (BCA) assay (Pierce). To analyze the PrP<sup>Sc</sup> content, 50 μg of protein was methanol precipitated. For PrPres, 250 μg of protein was digested with proteinase K (PK) under our standard conditions (4 μg of PK per 1 mg of protein, 2 h, 37°C) and further centrifuged at 22,000 × g for 30 min. The pellets were dissolved in 0.1 M solution sample buffer.

**Detection of PrP<sup>Sc</sup> and PrPres by immunoblotting.** Samples were loaded on a 12% Bis-Tris polyacrylamide gel (Invitrogen) and run in morpholineethanesulfonic acid (MES) buffer. Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) by semidry blotting using a Trans-Blot SD cell (Bio-Rad) as previously described (18). For PrP<sup>Sc</sup> detection, membranes were further incubated with the mouse monoclonal anti-protein antibody 4F2 (antitachyrepeat) and horseradish peroxidase-conjugated anti-mouse IgG as a secondary antibody. To detect PrPres, membranes were incubated with the biotinylated mouse monoclonal anti-protein antibody Sh3A1 (epitope from residue 145 to 152 [human numbering]) and then with peroxidase-conjugated streptavidin. Revelation was done using the BM chemiluminescence blotting substrate (Roche). Molecular weight markers were Precision Plus protein standards (Bio-Rad).

**Immunofluorescence and confocal microscopy.** After fixation with 4% paraformaldehyde, cells were permeabilized with 0.1% Triton X-100 or not as previously described (17). PrP<sup>Sc</sup> was detected using 4F2 antibody and Alexa Fluor 488-conjugated goat anti-mouse antibodies (Molecular Probes). For colocalization experiments, permeabilized cells were further incubated with a rabbit polyclonal anti-galactosyl antibody (Abcam) and Alexa Fluor 568-conjugated goat anti-rabbit as secondary antibody, whereas nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Sigma). To assess the presence of PrP<sup>Sc</sup> at the outer leaflet of the cell surface, adherent live cells were washed and labeled with 4F2 antibody and Alexa Fluor anti-mouse antibodies at 4°C in medium containing 1% heat-inactivated FCS. Images were acquired with a Zeiss Axiovert 200 M microscope and a charge-coupled device (CCD) cool-snap HQ camera (Roper...
Results

Expression and subcellular localization of “monoglycosylated” PrP mutants. We generated an initial series of PrP glycosylation mutants in which the asparagine residue of one each of the consensus sites (N184 and N200 in the ovine sequence) was replaced by one of the five amino acid residues D, H, S, T, and Q. The first four amino acids were selected because they are statistically associated with a lower propensity to modify the structure and/or function of proteins upon substitution (51); Q was used because this amino acid has often been chosen on a steric basis to suppress glycosylation sites (37). Expression vector constructs encoding mutant or wild-type PrP (VRQ allotype) were introduced into RK13 parental cells, and populations of stably transfected cells were selected. PrP expression in the “Rov” cell cultures thus obtained was induced by doxycycline and analyzed by immunoblotting (Fig. 1). The PrP mutants displayed the expected molecular profile, in which the upper, broad band corresponding to the diglycosylated species of N184X and N200X mutants was predominant (Fig. 1). The mobility of the glycosylated species appeared overall to be slightly lower for site 1 than that for site 2 mutants, consistent with the lower molecular mass of the glycan chain attached to site 1 (37).

Additional site 1 glycosylation mutants were produced by replacing T (instead of N) in the consensus sequon by either A or N, the glycoform pattern of which did not differ from that of N184X mutants (data not shown).

Prion formation and self-propagation in monoglycosylated PrP cells. Cells stably expressing mutant PrP were exposed to ovine prion strain 127S. All five N200 PrP mutants formed PK-resistant PrP\textsuperscript{Sc} (PrPres) from the first passage postexposure on, as well as on subsequent passages of the cultures (Fig. 5). This result, based on three independent experiments using at least two different cell populations produced through independent transfection and selection, was indicative of sustainable infection, as opposed to simple, acute conversion into PrP\textsuperscript{Sc}. However, the efficiency of conversion, in terms of the level and sustainability of PrPres accumulation, reproducibly differed among the mutants. Thus, while N200D cells accumulated nearly as much PrPres as wild-type Rov cells and were able to propagate the infection steadily for up to 10 consecutive passages (Fig. 5), N200H cells accumulated PrPres at much lower levels (Fig. 5) and not beyond...
the third or fourth subpassage. N200Q, N200S, and N200T cells accumulated at least 10-fold less PrPres than wild-type Rov cells, yet successful propagation occurred for up to 5 or 10 passages. Isolation and individual infection of cell subclones led to similar observations (data not shown).

Clearly different results were obtained when Rov cells expressing N184X PrP mutants were exposed to infection. Indeed, N184D was the only one of the site 1 mutants to show evidence of conversion into PrPSc at a substantial level (Fig. 5 and Table 1). Additional amino acid changes (not listed in Table 1) were tested, including E and G, which were expected to be good substitutes for D. In short, none of the nine mutants produced a detectable accumulation of PrPres, even at the first passage postexposure, except N184D. N184D Rov cell cultures were reproducibly infectible (in five independent experiments) and produced PrPres for at least five passages postexposure (Fig. 5). Cell subclones expressing N184D were isolated and also found to be readily infectible, but again this turned out to be unsuccessful with other mutants.

To confirm that ovine prions propagated in cells expressing monoglycosylated mutant PrPC were truly infectious, cell extracts were prepared from such cultures and then used as the inoculum for de novo infection experiments. As shown in Fig. 6, prions propagated in cell cultures expressing N200D were able to infect cultures expressing the homotypic as well as the wild-type PrP sequence. Similar results were obtained by using infecting prions derived from cells expressing N184D (not shown).

**Failure of unglycosylated PrP mutants to form PrPSc.** To produce unglycosylated double PrP mutants, we used constructs that combined the sole amino acid change found to be permissive at position 184 and position 200. These mutants will thereafter be designated using a shortened form, for instance, NDND instead of N184D-N200D. Additional mutants were designed, including NQNQ and NTNT, because these mutations had already been used in mouse transgenesis and some combinations of those and substitutions were used in previous studies. Altogether, 13

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**TABLE 1. Characteristics of the PrP glycosylation mutants**

<table>
<thead>
<tr>
<th>Glycotype</th>
<th>Genotype</th>
<th>Cell surface expressiona</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (VRQ)</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monoglycosylated site 1</td>
<td>N184D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mutants</td>
<td>N184H</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>N184Q</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>N184S</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>N184T</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>N184G</td>
<td>+</td>
<td>–</td>
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<td>N184E</td>
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<td>–</td>
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<tr>
<td></td>
<td>T186N</td>
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</tr>
<tr>
<td>Monoglycosylated site 2</td>
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</tr>
<tr>
<td>mutants</td>
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<tr>
<td></td>
<td>N200Q</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>N200S</td>
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<td>+</td>
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<tr>
<td></td>
<td>N200T</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Unglycosylated double</td>
<td>N184D-N200D</td>
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</tr>
<tr>
<td>mutants</td>
<td>N184D-N200H</td>
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<tr>
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<td>N184D-N200Q</td>
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<td>N184G-N200D</td>
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<td>–</td>
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<tr>
<td></td>
<td>N184T-N200T</td>
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a, +, ≥ 55%; –, ≤10% (relative to wild-type PrP).

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**FIG. 3.** Comparative cell surface expression of PrP glycosylation mutants as determined by flow cytometry. The expression levels of the mutants are provided as percentage relative to wild-type PrP (means and standard deviations from three experiments; 4F2 antibody). NT, nontransfected RK13 cells. G37T-NDND designates a double mutant with an extra glycosylation site, which is described in the text.

**FIG. 4.** Accumulation of PrPSc mutants in the Golgi compartment. PrP and giantin were labeled as for Fig. 2, and fluorescence emission was examined by confocal microscopy. Z-axis planes are shown. The mutants analyzed (D to O) are indicated on the left (for G37T-NDND, see the Fig. 3 legend). Bars, 10 μM.
double PrP mutants were generated (Table 1 and data not shown). As a common feature, such mutants were expressed at the cell surface very poorly, despite global expression levels approaching those of monoglycosylated mutants (Fig. 1). PrP immunofluorescent labeling was essentially intracellular and mostly colocalized with a marker of the Golgi complex, which is indicative of a markedly impaired trafficking (Fig. 2a and b). Upon exposure to infectious inoculum, the cultures expressing such mutants consistently failed to accumulate PrPres even transiently, despite repeated attempts.

**Restoration of cell surface expression by creation of an artificial glycosylation site.** In the experiments described above, none of the PrP mutants (15 out of 15) showing no or minimal cell surface expression was able to form PrPSc at a detectable level, suggesting that adequate trafficking to the plasma membrane is a key, if not an absolute requirement. Therefore, we asked whether introduction of an ectopic glycosylation site would restore the normal phenotype and, if so, the permissiveness. To this end, an NXT consensus sequon was created in the sequence of the NDND mutant by mutating codon 37 so as to change T to G, downstream from the first asparagine (N35) present in the N-terminal region of the molecule. Immunoblotting analysis showed that the G37T-NDND mutant PrP consisted mostly of glycosylated species, indicating that the newly introduced NXT sequon acted as an efficient sugar attachment site (Fig. 7a). As a control, the same mutation was introduced in the wild-type sequence. The PrP glycosylation pattern of the latter mutant (G37T) was modified compared to that of wild-type PrP, with the presence of a larger, presumably triply glycosylated species and also the nearly complete disappearance of the unglycosylated species, further confirming that the ectopic NTT codon was fully functional. The G37T and G37T-NDND mutants had similar subcellular distributions, and both were properly trafficked to the outer cell surface, as revealed by immunofluorescence labeling of live or fixed cells (Fig. 7b; see also Fig. 4M). Moreover, both mutants showed association with flotillin (Fig. 7c), which is considered a raft component and classically used as a marker for lipid microdomains. Upon exposure to prion, however, G37T-NDND turned out to be unable to form PrPSc, whereas G37T (with three glycan attachment sites) was converted as efficiently as wild-type PrP and allowed robust, sustainable infection (Fig. 7d). Similar results, i.e., efficient glycosylation and expression at the cell surface but lack of conversion into PrPSc, were obtained with two other mutants, G37T-T186N, and Y52T-NDND, in which the Y52T mutation created a novel consensus sequon (NRT) involving asparagine 50 (Table 2). From these results it was concluded that restoring the trafficking of a mutant PrP to the cell surface was not sufficient to confer on this mutant the ability to convert into PrPSc.

**GPI anchor-deficient PrP can form PrPSc upon infection.** The lack of a GPI anchor has been reported to perturb the process of glycosylation of proteins during their translocation in the endoplasmic reticulum (ER) (26). Indeed, GPI-deficient (ΔGPI) PrP consists mostly of unglycosylated species in mouse and in cell culture (14, 36, 61). Production of ΔGPI PrP was thus a possible means to gain further insight on the role of the glycan chains in prion biology in cultured cells. RK13 cells were transfected using a construct with a stop codon placed at the usual cleavage site of the GPI anchor signal peptide. As expected, ΔGPI PrP molecules displayed distinctive features compared to wild-type PrP (Fig. 8a and data not shown): (i) higher electrophoretic mobility, (ii) predominance of unglycosylated species and absence of diglycosylated ones, and (iii) lack of expression at the cell surface and release in the supernatant, with a small proportion of molecules remaining intracellular (not shown). In order to augment the concentration of the protein at the monolayer cell surface, prion-exposed cultures were maintained in agarose-containing semisolid medium, which was previously shown to have no adverse effect on prion propagation (43). These cultures were kept without splitting for 2 weeks, at which time the input PrPSc material was cleared and no longer detectable. Under these conditions, we were able to detect the conversion of the ΔGPI PrP into a PK-resistant form that was essentially unglycosylated, with a minority species corresponding to a monoglycosylated form (Fig. 8a). These results indicated that unglycosylated PrP with a wild-type core sequence can convert into PK-resistant PrP.
even when glycosylated species are largely underrepresented. The amount of protein converted was markedly lower than that of wild-type PrP, and PK-resistant \( /H9004 \) GPI PrP no longer accumulated after a subsequent passage of infected-cell culture overlaid or not with agarose. However, \( /H9004 \) GPI-PrP\( \text{Sc} \) was clearly infectious, since it was able to transmit infection to Rov9 cells (58) expressing wild-type PrP\( \text{C} \) (Fig. 8b).

FIG. 8. Ability of anchorless PrP to form PrP\( \text{Sc} \). (a) Left panel, immunoblot analysis of Rov cell lysates expressing \( /H9004 \) GPI or wild-type PrP\( \text{C} \); middle panel, PK sensitivity of \( /H9004 \) GPI PrP expressed in mock-infected cells; right panel, PK resistance of \( /H9004 \) GPI PrP in cultures exposed to infectious prion inoculum (see Materials and Methods; Sha31 antibody). None, noninfected, parental RK13 cells. Note the faster migration of \( /H9004 \) GPI (arrowheads) compared with wild-type PrP. Upper bands in the infected, PK-treated nontransfected, and \( /H9004 \) GPI samples represent nonspecific background. (b) \( /H9004 \) GPI PrP\( \text{Sc} \) is infectious. Cellular material prepared from lysates of infected cultures such as shown in panel a (either RK13 [none] or \( /H9004 \) GPI cells) was inoculated into cells expressing wild-type PrP\( \text{C} \). PK-treated samples from infected cells at the second passage are shown.

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FIG. 7. Expression and permissiveness to prion infection of PrP mutants with an extra glycosylation site. (a) Immunoblot showing the PrP\( \text{C} \) glycoform profiles of mutants bearing either three glycosylation sites (G37T) or the extra glycosylation site only (G37T-NDND). The artificial N-linked glycosylation sequon created by replacement of a glycine by a threonine at position 37 was truly functional, as evidenced by the modification of the glycoform profiles of G37T-NDND and of G37T mutants compared to NDND and wild-type PrP, respectively. (b) Expression of G37T-NDND PrP at the cell surface. Immunofluorescence on living cells using 4F2 antibody is shown. G37T and G37T-NDND PrPs show similar cell surface labeling, while the NDND mutant is essentially negative. Bars, 20 \( \mu \)M. (c) G37T-NDND mutant and wild-type PrPs are both associated with lipid rafts. An immunoblot analysis performed on step sucrose gradient fractions using anti-PrP and anti-flotillin antibodies is shown. Fractions 3 and 4 correspond to the interface of 5% and 30% sucrose cushions. (d) Permissiveness to de novo prion infection of the G37T PrP mutants. An immunoblot with PK-treated cell lysates from cultures at passage 3 postexposure is shown (Sha31 antibody). No PrPres signal is visible in G37T-NDND mutant-expressing cells, although G37T mutant and wild-type PrP-expressing cells accumulate PrPres at similar levels.

TABLE 2. Effect of ectopic glycosylation on PrP cell surface expression and conversion

<table>
<thead>
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<th>Cell surface expression(^a)</th>
<th>Conversion</th>
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<tr>
<td></td>
<td>N35</td>
<td>N50</td>
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</tr>
<tr>
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<td>Y52T-N184D-N200D</td>
<td>–</td>
<td>+</td>
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<tr>
<td>G37T-N184Q-N200Q</td>
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<td>G37T-N184T-N200T</td>
<td>+</td>
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<tr>
<td>T186N</td>
<td>–</td>
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<td>G37T-T186N</td>
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\(^a\) +, \( \geq 55\% \); –, \( \leq 10\% \) (relative to wild-type PrP).
PrP mutations associated with atypical PrP<sup>C</sup> or PrP<sup>Res</sup> glycoform ratios. Several mutations associated with genetic TSE diseases in humans are located within or in the close vicinity of a PrP glycosylation site. These include T183A, which was analyzed here in the ovine sequence context (T186A) (see above), and V180I and F198S, which were reported to modify the glycoform pattern of PrP<sup>C</sup> or PrP<sup>Res</sup> (13, 24, 63). We sought to examine the effect of the latter mutations on 127S prion propagation when engineered in the ovine PrP sequence. V183I ovine PrP<sup>C</sup> exhibited a normal glycoform pattern, similar to that of its human counterpart V180I PrP<sup>C</sup>. This mutation was permissive to infection (Fig. 9), but disappointingly, the PrPres generated did not show an atypical profile such as described in human brain tissue, which is characterized by the total absence of diglycosylated species (13). The F198S mutation, as well as the recently described F198V mutation (64), targets the central amino acid of the consensus sequon (NFT). The equivalent mutations in the ovine sequence, F201S and F201V, respectively, both induced altered glycoform profiles in which unglycosylated PrP<sup>C</sup> was essentially lacking (Fig. 9), similar to that reported for the human F198S PrP mutation (63). In Rov cells, both PrP mutants were properly expressed at the plasma membrane (not shown). However, upon exposure to infectious prions, these two mutations produced opposite effects, since F201S-engineered cells were refractory to infection while F201V conferred permissiveness.

**DISCUSSION**

**Glycosylation and PrP<sup>C</sup> trafficking.** Asn-linked carbohydrate chains play an important role in the intracellular trafficking and transport to the cell surface of many glycoproteins. Immunofluorescence analysis indeed showed that the subcellular localization of the ovine PrP<sup>VRQ</sup> glycosylation mutants was altered compared to that of the wild-type protein, although to a variable extent. Despite some intracellular retention, mostly in the Golgi apparatus (which was not seen with wild-type PrP<sup>VRQ</sup> molecules), all the monoglycosylated N184X and N200X mutants generated were efficiently expressed at the cell surface. Thus, even in the absence of endogenous diglycosylated species, one glycan chain suffices to ensure proper expression of PrP at the cell surface. Two monoglycosylated mutants, however, both produced by replacing T instead of N in the consensus sequon (T186N and T186A, which is homologous to a human PrP mutation [24]), exhibited a dramatically impaired trafficking, although they successfully passed the ER quality control given their massive accumulation in the Golgi apparatus. Similarly, the double nonglycosylated mutants were marginally expressed at the cell surface and localized mostly in the Golgi apparatus. These results were somewhat surprising since in N2A cells, the surface expression levels of T182N and of doubly mutated T182N-T198A mouse PrP were like that of wild-type PrP (38). The different cell type or PrP sequence context, i.e., murine versus ovine, could account for these discrepant observations. Alternatively, wild-type PrP molecules concomitantly expressed in the N2a cell system might exert some rescuing activity in trans. In one study, coexpression of wild-type and mutant prion proteins was found to alter their cellular localization and partitioning into membrane domains (52). In any case, it should be emphasized that the pattern of subcellular expression observed with nonglycosylated PrP mutants in the Rov cell system resembled that reported in neuronal cells in brain sections from knock-in mice that express the same mutation (9), thus arguing that Rov cells are a biologically relevant model for such studies.

A salient observation resulting from this study is that introduction of an Asn glycosylation site in the N-proximal, non-structured region of the molecule (e.g., mutation G37T) conferred double PrP glycosylation mutants a wild-type-like phenotype in terms of cell surface expression efficiency and membrane subdomain distribution. This implies that attachment of an ectopic glycan chain provided a signal in cis that sufficed to satisfy the Golgi compartment quality control. N glycosylation is regarded as a key determinant in the Golgian quality control of glycoproteins, including GPI-anchored proteins (57). Moreover, in Rov cells PrP is mostly expressed apically (44), and N-glycans are frequently involved in the apical sorting in polarized cells (42). Notwithstanding this, was the lack of a glycan chain the sole cause for the observed strong retention of the PrP double mutants in the Golgi compartment? Three comments can be made in this regard. (i) Intriguingly, the maturation of PrP molecules with mutations not targeting the glycosylation sequon, but also leading to Golgi retention, was recently reported to be favored by a short deletion in the N-terminal sequence (2), close to the ectopic glycan attachment sites of the G37T and Y52T mutants; thus, such a compensating effect cannot be excluded here. (ii) Whether or not wild-type, naturally aglycosylated PrP molecules undergo retention in the Golgi compartment is poorly documented. Mutated PrP molecules lacking a GPI anchor, almost exclusively aglycosylated, exit from the Golgi compartment and are secreted (14, 36, 60), but this may reflect a less stringent quality control of secreted compared to raft-associated proteins in the Golgi complex (22). Forward trafficking of PrP was reported to be unimpaired in tunicamycin-treated SH-SY5Y and CHO cells (31, 61), implying that aglycosylated PrP species can satisfy the Golgi quality control. Lastly, circumstantial data suggest that the glycoform ratio of cell surface-expressed PrP does not greatly differ from that of total PrP (33, 40, 55). (iii) Within cells where glycan synthesis takes place normally, glycosylated molecules may provide in trans a signal that favors the sorting of nonglycosylated molecules. In

FIG. 9. Permissiveness to prion infection of PrP mutants with an altered glycoform pattern. Human PrP mutations reported to alter the PrP<sup>C</sup> glycosylation pattern were introduced into the sheep sequence, as indicated (ovine PrP numbering). Immunoblot analysis for PrP<sup>C</sup> (a) (mock-infected culture, 4F2 antibody) and PrPres (b) (prion-infected cultures at third passage postexposure, Sha31 antibody) is shown. Note that the F210V mutant but not the F210S mutant conferred susceptibility to infection.

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support of such a “cooperative” scenario, oligomerization has been proposed to be a specific requirement for apical sorting of GPI-anchored proteins (41). Incidentally, it would also provide a simple explanation for the above-mentioned conflicting observations regarding the expression at the cell surface (38), or lack of such expression (reference 9 and this study), of glycosylated PrP mutants, depending on whether wild-type molecules are coexpressed or not in the cell system studied.

**Glycosylation and permissiveness to prion infection.** Three main findings arose from our experiments. First, all the monoglycosylated site 2 mutants (5/5) allowed de novo infection and prion propagation over several subpassages. In contrast, only one out of the nine mutations on site 1 tested in the infection assay led to sustained prion propagation. This result, while confirming the dispensability of either glycan chain, as also shown in vivo in mouse prion-infected transgenic mice (38, 56), underscores the possibility that any effect observed on prion biology could involve the amino acid substitution itself rather than simply the loss of the glycan chain. Indeed, the greater versatility of site 2 is likely to hold to the attachment of the glycan chain within the helix 2-helix 3 loop, whereas site 1 involves a more constrained domain of the PrP core, helix 2. Of note, two of the site 1 mutants (N184T and T186N) that were found to confer a decreased susceptibility to transgenic mice, in a strain-dependent manner (38, 56), failed to form PrPSc in the Rov cell system. Reciprocally, it would be relevant to investigate the effects of the N184D mutation in transgenic mice, because this substitution, which to our knowledge has rarely if ever been used to disrupt an Asn glycosylation sequon in a glycoprotein, was the only one that conferred permissiveness for de novo infection to a site 1 mutant in the present study. A drastic effect of the amino acid change was also observed with two mutations targeting the central residue of the consensus sequon: F201S and F201V mutants showed a similarly altered PrPSc glycoform pattern with a marked decrease of unglycosylated species, without alteration of the cell surface expression; however, F201V supported prion infection, whereas F201S did not.

Second, mutations that led to undetectable or hardly detectable expression at the cell surface were invariably associated (15 out of 15 mutants) with failure of the culture to propagate prions. This was notably the case for the monoglycosylated T186X mutants and for all the double mutants, even when the most favorable substitution (N to D) in terms of infectibility was used at both sequons. These results further strengthen the view that prior access of the PrP molecules to the cell surface is mandatory so that the conversion into PrPSc can take place (6, 12, 19, 23).

Third, stable and high-level cell surface expression of the glycosylation PrP mutant turned out not to be sufficient to allow prion propagation. As a matter of the fact, cell surface expression of six of the site 1 mutants found to be fully resistant to conversion (see above) did not greatly differ from that of wild-type molecule. In contrast, five of these same mutations were found to be permissive for transient conversion by mutation scanning in ScN2a (28). This suggests that coexpression with wild-type PrP and/or use of preinfected cell cultures is an experimental condition leading to an underestimation of the potential antagonistic effect of a mutation on prion propagation. Also supporting the contention that high-level surface expression was not sufficient, the double mutant NDND, the trafficking of which was restored by an ectopic, truly functional Asn glycosylation site (G37T; see above), did not allow prion multiplication either, whereas PrP molecules bearing both this extra site and the natural glycan attachment sites behaved like wild-type PrP in terms of efficiency and sustainability of the infection.

Strikingly enough, we failed at detecting even transient PrPSc accumulation in any of the nonpermissive, cell surface-expressed mutants, including G37T-NDND, indicating that acute conversion did not take place. This was not unexpected, as there is ample evidence that single-amino-acid differences in the PrP sequence may dramatically affect prion formation for a given strain both in vitro and in vivo (34, 46). In contrast, acute conversion (not sustained propagation) was repeatedly observed in Rov cultures expressing PrP lacking a GPI anchor, mostly consisting of aglycosylated species, corroborating a recent study on cells that expressed mouse anchorless PrP (36). Various lines of evidence in vivo and in vitro tend to suggest that a glycosylation defect does not necessarily impair, or could even ease, PrP conversion (7, 14, 30, 39, 47, 54, 62). Taken together, these observations lend support to the view that the inability of aglycosylated mutants that satisfied various cell sorting quality controls to convert into PrPSc might involve conformational dynamics of the mutant molecules, in turn affecting the efficiency of conversion process.

In conclusion, the results of this study with the Rov cell culture model established that not only the subcellular trafficking of mutant molecules but also the ability of properly trafficked molecules to form PrPSc can be variably altered, depending on the amino acid change introduced. The possibility is therefore real, not just theoretical, that any effect observed in vivo on the propagation of the agent in mice expressing PrP with a mutated glycosylation sequon(s) could actually be unrelated to the glycan chain deficiency per se. This issue deserves greater consideration, all the more because for obvious technical reasons only a few such mutations have been studied by mouse transgenesis. Much deeper investigations will probably be needed to understand the exact role of the Asn-linked carbohydrate moiety of PrP in prion biology.

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