

Expression of unglycosylated mutated prion protein facilitates PrP^{Sc} formation in neuroblastoma cells infected with different prion strains

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Prion replication involves conversion of the normal, host-encoded prion protein PrP^C, which is a sialoglycoprotein bound to the plasma membrane by a glycoposphatidylinositol anchor, into a pathogenic isoform, PrP^{Sc}. In earlier studies, tunicamycin prevented glycosylation of PrP^C in scrapie-infected mouse neuroblastoma (ScN2a) cells but it was still expressed on the cell surface and converted into PrP^{Sc}; mutation of PrP^C at glycosylation consensus sites (T182A, T198A) produced low steady-state levels of PrP that were insufficient to propagate prions in transgenic mice. By mutating asparagines to glutamines at the consensus sites, we obtained expression of unglycosylated, epitope-tagged MHM2PrP(N180Q,N196Q), which was converted into PrP^{Sc} in ScN2a cells. Cultures of uninfected neuroblastoma (N2a) cells transiently expressing mutated PrP were exposed to brain homogenates prepared from mice infected with the RML, Me7 or 301V prion strains. In each case, mutated PrP was converted into PrP^{Sc} as judged by Western blotting. These findings raise the possibility that the N2a cell line can support replication of different strains of prions.

Introduction

Prion diseases are transmissible neurodegenerative disorders of humans and animals (Prusiner, 1998). Prions consist of a pathogenic isoform (PrP^{Sc}) of a normal membrane-anchored host protein (PrP^C) and replication involves the formation of PrP^{Sc} from PrP^C. PrP^{Sc} acts as a template in the conversion process and an as yet unidentified molecule, designated protein X, is an important participant (Kaneko *et al.*, 1997; Telling *et al.*, 1995; Zulianello *et al.*, 2000). PrP^C is a 33–35 kDa glycoposphatidylinositol-anchored membrane protein with two N-linked glycosylation sites at residues 180 and 196 in mouse prion protein (Endo *et al.*, 1989; Lochter *et al.*, 1986). The glycosylation sites are variably occupied, and typically four variants of prion protein co-exist: the double-glycosylated, two monoglycosylated and the unglycosylated. Subtle differences in the glycan content between PrP^C and

PrP^{Sc} have been found recently, arguing that the glycosylation machinery may be perturbed in PrP^{Sc}-forming cells (Rudd *et al.*, 1999).

When different strains of prions are propagated within the same species, they are known to retain their original properties (Bruce & Dickinson, 1987; Dickinson & Meikle, 1969; Pattison & Millson, 1961). These strains are described by characteristic neuropathological patterns of plaque deposition, vacuolation, neuronal loss and astrogliosis, as well as different incubation times and, in part, different biochemical characteristics (Bessen & Marsh, 1994; Safar *et al.*, 1998; Scott *et al.*, 1997).

In the absence of an identifiable nucleic acid in highly purified prion preparations (Kellings *et al.*, 1994), differences between strains can only be explained by a replicative mechanism that occurs post-translationally. Strong evidence suggests that strain characteristics are enciphered in the conformation of PrP^{Sc} itself (Bessen & Marsh, 1994; Safar *et al.*, 1998; Scott *et al.*, 1997; Telling *et al.*, 1996). The molecular mechanism of replication of strain-specific features through modulation of PrP^{Sc} conformation is not yet understood. Distinct prion isolates induce accumulations of PrP^{Sc} in the brain that are region-specific (Bruce *et al.*, 1989). This fact has

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led to the hypothesis that distinct cell types in the central nervous system dictate the strain-specific traits enciphered in the specific structure of each PrP^{Sc} (Hecker *et al.*, 1992).

Another hypothesis concerning strain replication is based on the observation that strains of prions seem to maintain a distinctive pattern of PrP^{Sc} glycosylation site occupancy (Collinge *et al.*, 1996; Parchi *et al.*, 1997). From these findings, it has been hypothesized that strain characteristics are encrypted in their glycosylation patterns as assessed by the banding patterns of proteinase K-digested PrP^{Sc} on SDS-PAGE gels (Collinge *et al.*, 1996). However, glycoform patterns of PrP^{Sc} have been found to differ when comparing infected tonsil and brain tissues from the same Creutzfeldt-Jakob diseased patient (Hill *et al.*, 1999), spleen and brain samples from infected mice (Rubenstein *et al.*, 1991) and different brain regions within the same mouse brain (Somerville, 1999). Also, the passaging of different mouse prion strains in N2a cells overexpressing mouse PrP was shown to alter the glycosylation patterns of PrP^{Sc} (Nishida *et al.*, 2000), arguing that glycosylation is unlikely to specify strain-specific properties of prions.

In addition to the studies noted above that question the proposed role of glycoforms in prion strains, investigations of fatal insomnia argue persuasively that strain-specified properties are not enciphered in the asparagine-linked oligosaccharides (Mastrianni *et al.*, 1999; Parchi *et al.*, 1999). The familial form of fatal insomnia (FFI) is a genetic prion disease linked to a mutation of aspartate to asparagine at residue 178 (D178N) and a methionine polymorphism at residue 129 in the human prion protein (Goldfarb *et al.*, 1992; Lugaresi *et al.*, 1986). The disease phenotype of FFI is sleep loss and thalamic degeneration. Recently, patients have been described with phenotypes indistinguishable from FFI, but without any mutation in the PrP gene. These patients carry the diagnosis of sporadic fatal insomnia (sFI) (Mastrianni *et al.*, 1999; Parchi *et al.*, 1999). Moreover, patients with sporadic and familial forms of fatal insomnia exhibit a prion disease phenotype that is indistinguishable even though the levels of di- and monoglycosylated PrP^{Sc} molecules are markedly different (Mastrianni *et al.*, 1999). Additionally, extracts from the brains of patients with either the sporadic or familial forms of fatal insomnia transmit disease to transgenic mice with similar incubation times and neuropathological lesion profiles (Mastrianni *et al.*, 1999). The foregoing studies argue that although some strains may be correlated with certain glycosylation patterns, these patterns are inconsistent and cannot encipher strain-specific properties.

In this study, we investigated the influence of glycosylation on the conversion of PrP^C by mouse prion strains. While it has been shown that inhibition of glycosylation by tunicamycin in scrapie-infected cells results in protease-resistant unglycosylated PrP^{Sc} (Lehmann & Harris, 1997; Taraboulos *et al.*, 1990), attempts to express a metabolically stable, mutated unglycosylated PrP^C have been unsuccessful (DeArmond *et al.*,

1997; Lehmann & Harris, 1997; Rogers *et al.*, 1990). The present study introduces a novel mutation, replacing the asparagines (mouse PrP codons 180 and 196) which carry the carbohydrate side-chains with glutamines (N180Q,N196Q), rather than mutating threonines in the glycosylation consensus sequence (T182A,T198A) as previously described (DeArmond *et al.*, 1997; Lehmann & Harris, 1997; Taraboulos *et al.*, 1990).

Methods

■ **Cell culture.** Neuroblastoma cells (N2a) were purchased from the ATCC (Neuro-2a; CCL131) and subcloned (Bosque & Prusiner, 2000). The subclones were grown in MEM medium (Gibco Lifesciences) supplemented with 10% foetal calf serum and 10 mM penicillin-streptomycin. Permanently scrapie-infected neuroblastoma cells (ScN2a) derive from infection of neuroblastoma cells with the RML scrapie strain (Chandler, 1961), and subsequent subcloning (Bosque & Prusiner, 2000).

■ **Vectors.** Expression of recombinant epitope-tagged PrP using the CMV promoter-based pSPOX vector in N2a cells has been described (Kaneko *et al.*, 1997; Scott *et al.*, 1992). Introduction of the epitope for monoclonal antibody (MAb) 3F4 (Kasczak *et al.*, 1987) into mouse PrP (designated MHM2PrP) allows detection of newly formed PrP^{Sc} against the background of cell- and inoculum-resident PrP^{Sc}, which is not recognized by MAb 3F4 (Scott *et al.*, 1992). Although mutations at residues constituting the MAb 3F4 epitope have been implicated in altering the species barrier between prions (Priola *et al.*, 1994), the MHM2PrP construct has not been found to exert a significant impact on the susceptibility to mouse prions when compared to mouse PrP^{Sc} (Scott *et al.*, 1993). Generation of pSPOX vectors containing the 3F4 epitope and the Q218K mutation have been described (Kaneko *et al.*, 1997; Scott *et al.*, 1992). Mutations at codons 180 and 196 were introduced using mismatched primers in PCR of particular PrP templates. The resulting fragments were substituted into unique cloning sites of pSPOX. Specifically, primers 5' GGCAGATCTACCATGGCGAACCTTGGC 3' (sense) and 5' GTGGTGGTGGTGACCGTGTGCTGCTTGATGGTG-ATCTGGACCGCAGTC 3' (antisense) were used to create a fragment in which codon 180 was mutated to glutamine. The fragment was then cloned into unique sites *Bgl*III and *Bst*EII. Primers 5' CAGCACACCGT-CACCACCACCAAGGGGGAGCAGTTCACCGAG 3' (sense) and 5' CACTATAGAAGCTCGAGCAGCCTCCCT 3' (antisense) were used to create a fragment in which codon 196 was mutated to glutamine. The fragment was then cloned into unique sites *Bst*EII and *Xho*I. The correct nucleotide sequence of constructs was verified by sequencing the pSPOX inserts with an ABI prism 377 sequencer.

■ **Transient transfection of ScN2a cells.** A confluent 100 mm dish of ScN2a cells was washed, detached with 0.05% trypsin and resuspended in a total volume of 5 ml. These detached ScN2a cells were added in 0.5 ml aliquots to a 60 mm dish filled with 2 ml of fresh MEM medium. pSPOX vector (15 µg) carrying the respective PrP constructs was resuspended in DOTAP (Roche) according to the manufacturer and applied to each 60 mm dish of freshly split ScN2a cells; the next day, another 2 ml of fresh MEM medium was added. Four days after transfection, cells were harvested after being washed three times in PBS with 0.5 ml lysis buffer (150 mM NaCl, 10 mM Tris pH 8.0, 0.5% NP-40, 0.5% deoxycholate) per 60 mm dish. Lysate (0.4 ml) was digested with 20 µg/ml proteinase K (Roche) for 30 min at 37 °C. The digested lysate was then ultracentrifuged at 100 000 g for 45 min in a TLA 55 rotor

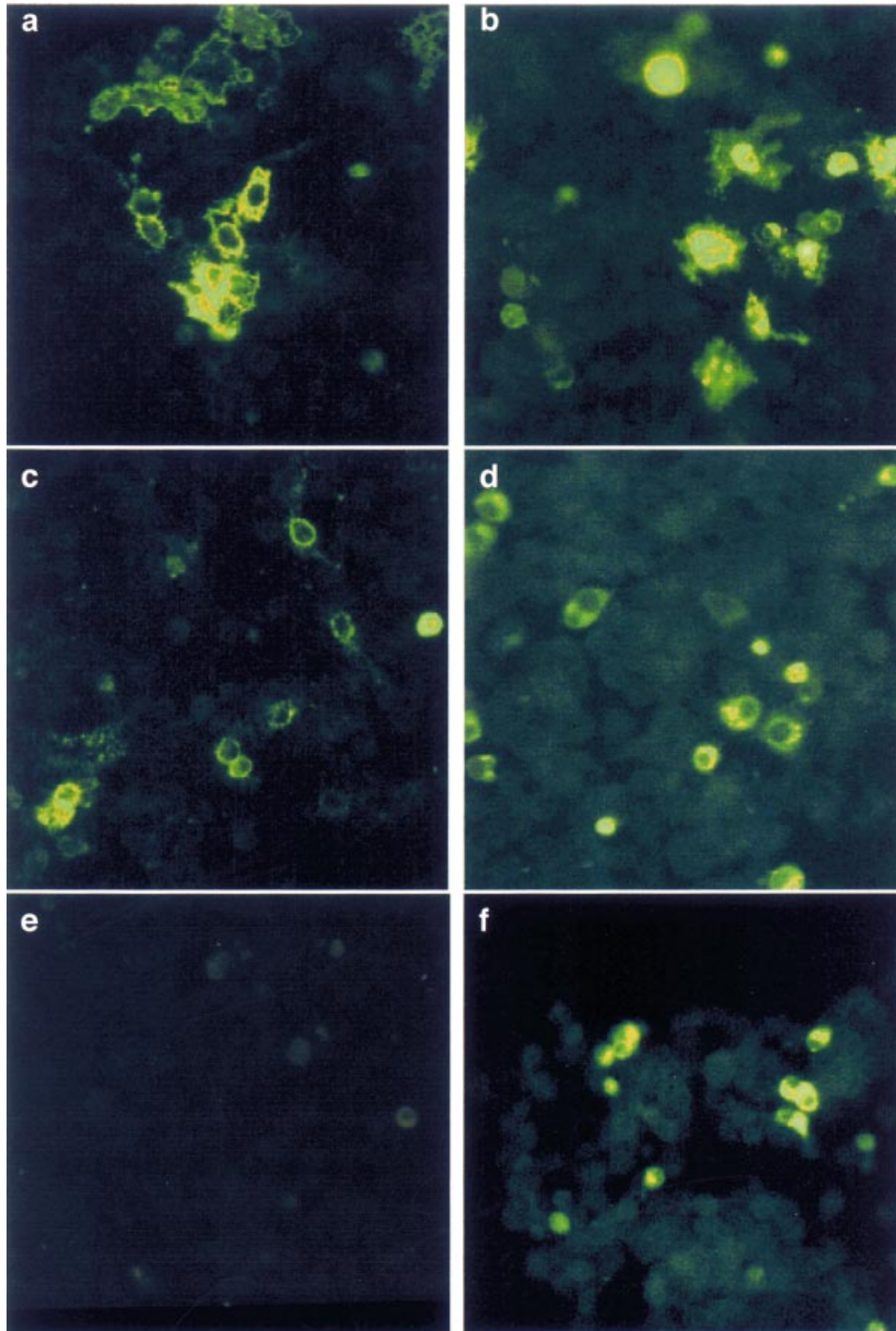


Fig. 1. Surface or intracellular immunostaining of transiently transfected ScN2a cells expressing the following constructs: (a) MHM2PrP surface staining, (b) MHM2PrP intracellular staining, (c) MHM2PrP(N180Q,N196Q) surface staining, (d) MHM2PrP(N180Q,N196Q) intracellular staining, (e) MHM2PrP(T182A,T198A) surface staining, (f) MHM2PrP(T182A,T198A) intracellular staining. Whereas both MHM2PrP and MHM2PrP(N180Q,N196Q) are stained on the cell surface (a, c) as well as in intracellular compartments (b, d), MHM2PrP(T182A,T198A) is positive for intracellular staining (f) but not for cell surface staining (e), suggesting that the latter construct is either retained in intracellular compartments or rapidly degraded on the cell surface.

(Beckman table-top ultracentrifuge). The supernatant was discarded and the pellet was resuspended in SDS-PAGE sample buffer. Finally, all samples were processed for Western blotting using standard procedures. The primary antibodies used were MAb 3F4 (Kascsak *et al.*, 1987) or Ro73. The secondary antibodies were HRP-labelled goat anti-mouse IgG and goat anti-rabbit IgG, respectively. The ECL/Hyperfilm detection system was obtained from Amersham. All experiments were repeated independently at least three times.

Immunofluorescence labelling. ScN2a cells were transfected as described above in dishes containing coverslips. Four days after transfection, coverslips were processed for surface staining and intracellular staining. For surface staining, coverslips were washed three times with cold PBS and then incubated for 1 h at 4 °C with MAb 3F4 diluted 1:50 with 1% BSA. Subsequent steps were performed at room temperature. Coverslips were washed again with PBS and fixed with 4% paraformaldehyde for 30 min. After blocking with 5% milk, 1% BSA in PBS, coverslips were washed in PBS and goat anti-mouse IgG-FITC-labelled secondary antibody (Roche) was added at a dilution of 1:100 for 30 min. Intracellular staining was carried out at room temperature as follows: coverslips were washed, incubated in 4% paraformaldehyde for 30 min and blocked with 5% milk, 1% BSA, 0.5% saponin for 30 min. Coverslips were then incubated in MAb 3F4 diluted 1:50 with 1% BSA, 0.5% saponin for 1 h. After washing in PBS, goat anti-mouse IgG-FITC-labelled secondary antibody (Roche) was added at a dilution of 1:100 for 30 min. All coverslips were finally washed three times in PBS, mounted on a glass slide with 5 µl mounting medium (Vetashield) and examined under a Leitz microscope.

Inoculation of transiently transfected N2a cells. Homogenates (10%) of whole mouse brains in PBS were prepared by passing them five times through successively smaller syringe needles from 16 to 26 gauge. The origins of the strains RML (Chandler, 1961), Me7 (Dickinson & Meikle, 1969) and 301V (Farquhar *et al.*, 1996) have been described. Homogenates were kept at -80 °C.

Confluent N2a cells on a 10 cm dish were split into five 10 cm dishes; 37 µg of pSPOX encoding different MHM2PrP constructs was suspended in 300 µl sterile 20 mM HEPES pH 7.5, and an equal volume of 20 mM HEPES pH 7.5 DOTAP (Roche) was added, incubated for approximately 12 min and added to the freshly split neuroblastoma cells. After 24 h, 150 µl of a 10% brain homogenate in PBS was added. Fresh MEM medium was added 24 h and 72 h after transfection. Five days after transfection (4 days after inoculation) cells were washed three times in PBS, lysed in 1 ml lysis buffer and processed for Western blotting as described above. All experiments were repeated independently at least three times.

Results

Unglycosylated MHM2PrP(N180Q,N196Q) is expressed on the cell surface of ScN2a cells

After transient transfection of pSPOX encoding MHM2PrP(N180Q,N196Q) into permanently scrapie-infected neuroblastoma cells (ScN2a), MHM2PrP(N180Q,N196Q) was expressed on the cell surface (Fig. 1c). MAb 3F4 stained the surfaces of ScN2a cells transiently transfected with MHM2PrP (Fig. 1a) and MHM2PrP(N180Q,N196Q) (Fig. 1c), but not MHM2PrP(T182A,T198A) (Fig. 1e). After permeabilization of the cells with saponin, intracellular compartments were stained with MAb 3F4 for all epitope-tagged

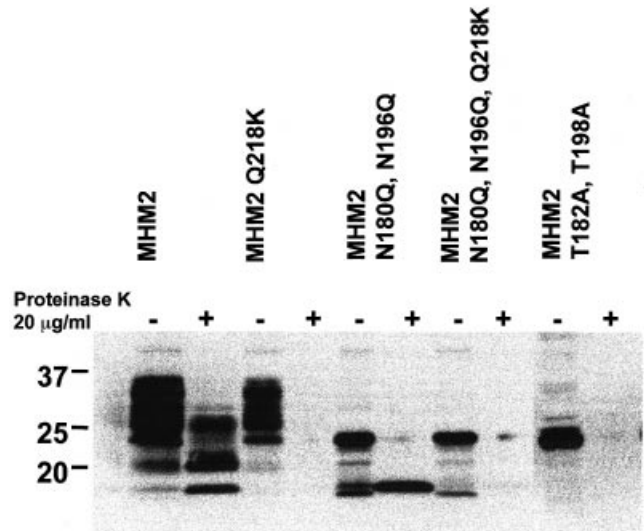


Fig. 2. Western blot of the expression and conversion of unglycosylated mutated PrP in ScN2a cells. Transiently transfected ScN2a cells express MHM2PrP(N180Q,N196Q) with an expected gel mobility of 24 kDa, which is converted into MHM2PrP^{Sc}(N180Q,N196Q) (gel mobility of 16 kDa), as is MHM2PrP. MHM2PrP(T182A,T198A) is not as efficiently converted. Addition of mutation Q218K to MHM2PrP(N180Q,N196Q) abolishes conversion, as it does in MHM2PrP.

constructs: MHM2PrP (Fig. 1b), MHM2PrP(N180Q,N196Q) (Fig. 1d) and MHM2PrP(T182A,T198A) (Fig. 1f). These findings demonstrate that by mutating the glycosylation consensus sequence by changing asparagine to glutamine (at codons 180 and 196), rather than threonine to alanine (at codons 182 and 198), an unglycosylated PrP is created that is correctly trafficked to the cell surface. The construct MHM2PrP(T182A,T198A) expressed in eukaryotic cells is unglycosylated and does not reach the cell surface (Fig. 1e). Interestingly, MHM2PrP(T182A,T198A) does seem to be retained within intracellular compartments (Fig. 1f). These results are in accordance with previous findings which demonstrated that PrP(T182A,T198A) accumulated prior to the mid-Golgi stack (DeArmond *et al.*, 1997; Lehmann & Harris, 1997; Rogers *et al.*, 1990). Of note, constructs with a single glycosylation site mutated [(MHM2PrP(N180Q) or MHM2PrP(N196Q)] were also trafficked to the cell surface (data not shown).

Unglycosylated MHM2PrP^c(N180Q,N196Q) is converted to protease-resistant MHM2PrP^{Sc}(N180Q,N196Q)

In ScN2a cells, MHM2PrP(N180Q,N196Q) was efficiently converted into protease-resistant PrP^{Sc}, whereas MHM2PrP(T182A,T198A) was not (Fig. 2). MHM2PrP^{Sc} usually shifts in its gel mobility after proteinase K digestion from 35–24 kDa to 27–16 kDa (Fig. 2, two left lanes); an

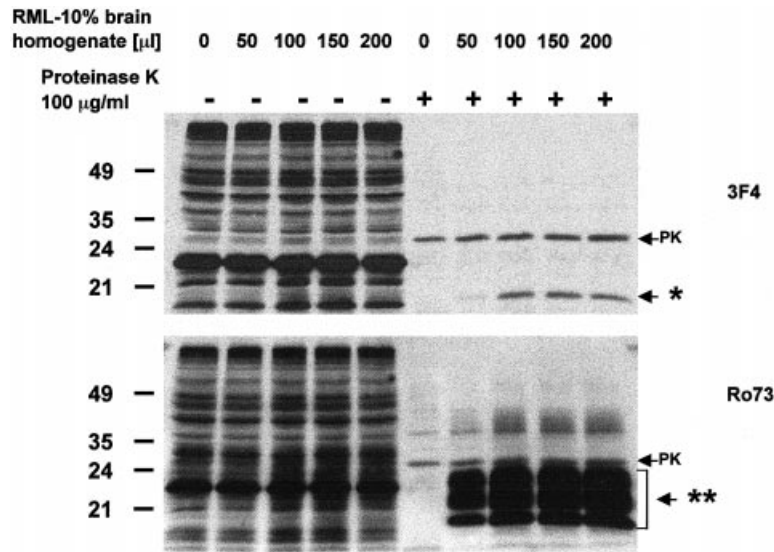


Fig. 3. Western blot presenting the conversion of transiently expressed unglycosylated MHM2PrP(N180Q,N196Q) by brain homogenates in N2a cells. N2a cells were transiently transfected with MHM2PrP(N180Q,N196Q) and inoculated with various quantities of a 10% RML-infected mouse brain homogenate. The five left lanes show lysates without proteinase digestion; the five right lanes show the same lysates after proteinase K digestion. Background for undigested lysates is high due to overexposure of the film to detect relatively weak protease-resistant unglycosylated MHM2PrP(N180Q,N196Q) (*). Therefore, cross-reactivity is visible between the secondary antibody and cell proteins in the undigested samples on the left side of the immunoblot, and a cross-reactive band of proteinase K (PK) appears in the digested lysates on the right. Expression levels of MHM2PrP(N180Q,N196Q) are about the same (determined by a shorter exposure time of the film for undigested lysates). The upper blot shows an immunoblot with MA b 3F4, which recognizes only newly synthesized MHM2PrP(N180Q,N196Q) (*). Addition of 100 μ l of 10% RML-infected brain homogenate was sufficient to convert MHM2PrP(N180Q,N196Q) into MHM2PrP^{Sc}(N180Q,N196Q). The lower panel shows the same blot stained with polyclonal antibody Ro73, which recognizes all PrP, and is used to show that equal amounts of inoculum (***) were present. The inoculum cannot be entirely washed out before lysing the cells.

analogous shift is seen with unglycosylated MHM2PrP^{Sc}(N180Q,N196Q) from a single 24 kDa band to a 16 kDa band (Fig. 2, lanes 5 and 6 from left). Both constructs migrated with an apparent molecular mass of 24 kDa as expected, indicating that neither was glycosylated (Fig. 2). The apparently inefficient formation of MHM2PrP^{Sc}(T182A,T198A) is consistent with findings from transgenic mice expressing MHM2PrP-(T182A,T198A) on the PrP knockout background, which could not be infected with mouse prions (DeArmond *et al.*, 1997).

Human prion propagation is inhibited in wild-type mice transgenic for human PrP; however, PrP^{0/0} mice that express human PrP transgenes do propagate human prions (Telling *et al.*, 1995). These observations led to the hypothesis that the products of the human PrP transgene and the mouse PrP gene are competing for the same conversion cofactor, provisionally termed protein X (Telling *et al.*, 1995). Systematic mutagenesis of PrP residues suspected of binding to protein X led to the identification of glutamine-218 in the mouse PrP sequence, which when mutated to lysine inhibited formation of protease-resistant PrP^{Sc} (Kaneko *et al.*, 1997; Zulianello *et al.*, 2000). This event is thought to happen through sequestration of the conversion cofactor, protein X, and to explain dominant negative inhibition of PrP^{Sc} formation. Since conversion of

MHM2PrP(N180Q,N196Q,Q218K) to PrP^{Sc} was also inhibited in ScN2a cells (Fig. 2), we infer that conversion of MHM2PrP(N180Q,N196Q), as for MHM2PrP, is an active process mediated by protein X, rather than a condition whereby MHM2PrP(N180Q,N196Q) is protected from protease by complexing to resident PrP^{Sc}. ScN2a cells transfected with single glycosylation site mutated PrP constructs [MHM2PrP-(N180Q) or MHM2PrP(N196Q)] both produced their protease-resistant counterparts; the Q218 mutation prevented the conversion of the single glycosylation site mutant MHM2PrP into MHM2PrP^{Sc} (data not shown).

Neuroblastoma cells transiently expressing unglycosylated MHM2PrP(N180Q,N196Q) can be infected with mouse prions from brain homogenates

Based on the results in ScN2a cells (Figs 1 and 2), a protocol was developed to infect transiently transfected N2a cells expressing MHM2PrP(N180Q,N196Q) with 10% scrapie-infected mouse brain homogenates. Four days after the inoculation of N2a cells expressing MHM2PrP(N180Q,N196Q) with 10% brain homogenate, newly formed MHM2PrP^{Sc}(N180Q,N196Q) could be detected by its epitope tag using

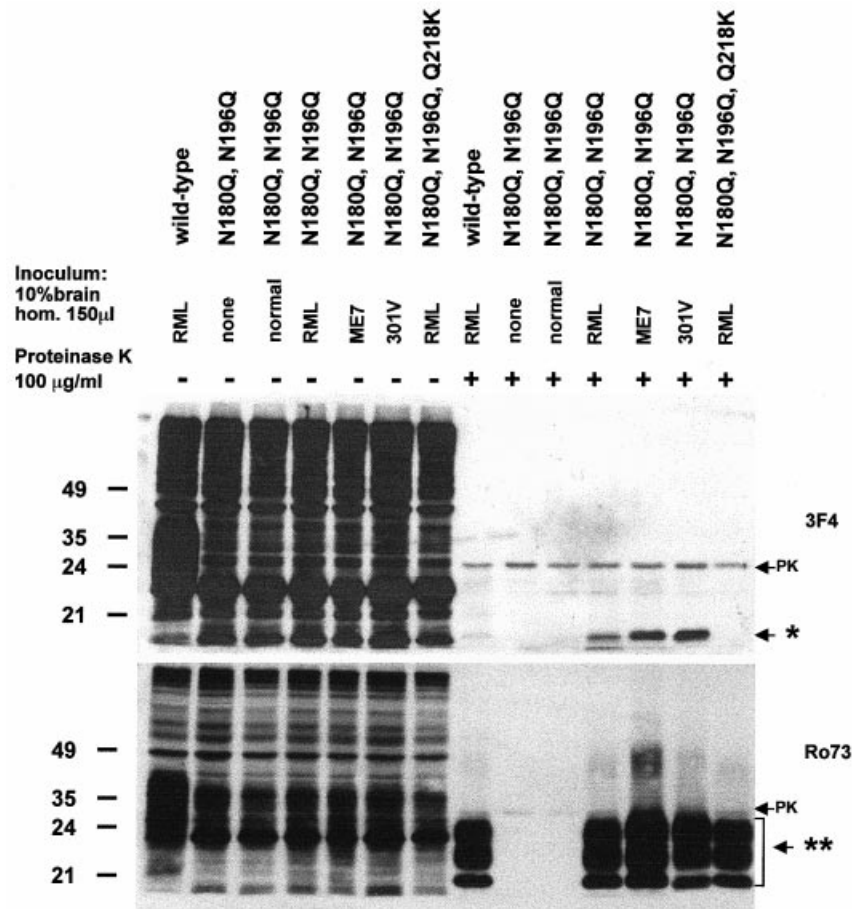


Fig. 4. Western blot presenting the conversion of transiently expressed unglycosylated MHM2 PrP(N180Q,N196Q) by different strains of mouse prions in N2a cells. The seven left lanes show lysates without proteinase digestion; the seven right lanes show the same lysates after proteinase K digestion. Background of undigested lysates is high due to overexposure of the film to detect relatively weak protease-resistant unglycosylated MHM2PrP(N180Q,N196Q) (*). Therefore, cross-reactivity is visible between the secondary antibody and cell proteins in the undigested samples on the left side of the immunoblot, and a cross-reactive band of proteinase K (PK) appears in the digested lysates on the right. Expression levels of MHM2PrP(N180Q,N196Q) are about the same as determined by a shorter exposure time of the film for undigested lysates. N2a cells were transiently transfected with different MHM2 PrP constructs as indicated and inoculated with an identical quantity (150 µl) of different mouse brain homogenates. The upper blot shows an immunoblot with MAb 3F4, which recognizes only newly synthesized MHM2PrP constructs. Inoculation of MHM2PrP(N180Q,N196Q)-expressing N2a cells with 10% brain homogenates of different mouse prion strains, RML, Me7 and 301V, converted MHM2PrP(N180Q,N196Q) equally well to MHM2PrP^{Sc}(N180Q,N196Q) (*). Neither the negative control (no inoculation) nor the control inoculation with normal CD-1 brain homogenate converted PrP(N180Q,N196Q). Also, inoculation of MHM2PrP-expressing N2a cells did not result in a significant conversion; a faint band with the electrophoretic mobility of unglycosylated PrP^{Sc} can be seen after proteinase K digestion, indicating that the unglycosylated population of PrP glycoforms is most readily converted into PrP^{Sc}. As for ScN2a cells (see Fig. 2), addition of another mutation, Q218K, to MHM2PrP(N180Q,N196Q) blocked conversion. The lower panel shows the same blot stained with polyclonal antibody Ro73, which recognizes all PrP and is used to show that equal amounts of inoculum (***) were present. The inoculum cannot be entirely washed out before cell lysis.

MAb 3F4. As shown in Fig. 3, 100 µl of 10% RML-infected mouse brain homogenate was sufficient to stimulate conversion of PrP(N180Q,N196Q) expressed in N2a cells into PrP^{Sc} after 4 days. Because the immunoreactive band at 16 kDa corresponding to protease-resistant MHM2PrP(N180Q,N196Q) is weak compared to undigested MHM2PrP(N180Q,N196Q), the immunoblots were overexposed (Figs 3 and 4). The overexposure increased both the PrP^{Sc} and background bands due to cross-reactivity with the secondary antibody, as can be

seen in the undigested lysates on the left side of the immunoblot and the cross-reactive band of proteinase K in the digested lysates.

Neuroblastoma cells transiently expressing unglycosylated MHM2PrP(N180Q,N196Q) can be infected with different mouse prion strains

Incubation conditions for transiently transfected neuroblastoma cells were varied: 150 µl of 10% brain homogenates

from mice infected with the RML (Chandler, 1961), Me7 (Dickinson & Meikle, 1969) or 301V (Farquhar *et al.*, 1996) strains of mouse scrapie all provoked MHM2PrP^{Sc}(N180Q, N196Q) formation after 4 days of incubation, whereas the no-inoculum control, or a 10% brain homogenate of a normal CD-1 mouse, did not stimulate PrP^{Sc} formation (Fig. 4). Wild-type MHM2PrP and MHM2PrP(N180Q, N196Q, Q218K) could not be converted as efficiently into PrP^{Sc} at 4 days after incubation with a 10% RML-infected mouse brain homogenate (Fig. 4). In fact, a faint band with the electrophoretic mobility of unglycosylated PrP^{Sc} can be seen after incubation of RML homogenate with N2a cells transiently transfected with MHM2PrP, indicating that unglycosylated PrP is most readily converted into PrP^{Sc}. Although the RML strain appears to convert MHM2PrP(N180Q, N196Q) less efficiently than the Me7 or 301V strain, as the band of protease-resistant MHM2PrP(N180Q, N196Q) is weaker than the two others, no definitive statement can be made since the inocula were not examined for the amount of PrP^{Sc} present, and the infectivity titres were not determined.

Discussion

The results presented here demonstrate that unglycosylated MHM2PrP(N180Q, N196Q) is correctly trafficked to the cell surface of neuroblastoma cells and converted to MHM2PrP^{Sc}(N180Q, N196Q) based on the acquisition of resistance to limited proteolysis as early as 4 days after exposure to prions from brain homogenates. These findings suggest that the construct MHM2PrP(N180Q, N196Q) may represent an improvement over the earlier construct MHM2PrP(T182A, T198A), which is unglycosylated but retained in intracellular compartments rather than correctly trafficked to the cell surface (Rogers *et al.*, 1990; Lehmann & Harris, 1997). The correct cellular trafficking of MHM2PrP(N180Q, N196Q) but not MHM2PrP(T182A, T198A) demonstrates also that the lack of glycosylation per se does not prevent PrP from being targeted to the cell surface, consistent with tunicamycin experiments. Presumably, localization of MHM2PrP(T182A, T198A) to intracellular compartments renders it less available for conversion to MHM2PrP^{Sc}(T182A, T198A) (DeArmond *et al.*, 1997).

An homogenate of scrapie-infected brain was sufficient to induce conversion of MHM2PrP(N180Q, N196Q) that was transiently expressed in neuroblastoma cells. In the inoculation protocol, no purification or concentration steps for prions in the inoculum were necessary to achieve MHM2PrP^{Sc}(N180Q, N196Q) formation after 4 days of incubation. The low efficiency of glycosylated PrP conversion compared to that of unglycosylated PrP is consistent with data showing that detection of mouse PrP^{Sc} in non-transfected N2a cells after inoculation with prions takes several weeks (Bosque & Prusiner, 2000; Butler *et al.*, 1988). Apparently, glycosylation

of asparagines 180 and 196 delays conversion of wild-type PrP^C to PrP^{Sc}, a conclusion that is consistent with earlier findings (Taraboulos *et al.*, 1990). Inhibition of PrP(N180Q, N196Q, Q218K) conversion by RML-infected brain homogenates demonstrates that the acquisition of protease resistance is an active process rather than protection of expressed PrP from protease digestion.

That transiently expressed MHM2PrP(N180Q, N196Q) is converted into PrP^{Sc} by different strains of mouse prions contrasts with infection of non-transfected cell lines (Bosque & Prusiner, 2000; Butler *et al.*, 1988; Race *et al.*, 1987; Schätzl *et al.*, 1997). We did not see any strain specificity with respect to the susceptibility of MHM2PrP(N180Q, N196Q) to conversion into PrP^{Sc}; however, we do not know if strain-specific characteristics were conserved during MHM2PrP^{Sc}(N180Q, N196Q) formation. Notably, we did find similar levels of PrP^{Sc} formation during inoculation with RML, Me7 or 301V. Although Me7 and RML have similar incubation times in CD-1 mice of approximately 150 days, 301V prions have an incubation time of approximately 230 days. These findings suggest that multiple prion strains more readily convert unglycosylated than glycosylated PrP^C in ScN2a cells into PrP^{Sc} and that strain-specified properties of prions do not alter the conversion of MHM2PrP(N180Q, N196Q) into PrP^{Sc}. It is noteworthy that other investigators have found that N2a cells overexpressing wild-type mouse PrP are more susceptible to prion infection than untransfected cells (Nishida *et al.*, 2000). They also reported that the Chandler, 139A and 22L prion strains infected these N2a cells overexpressing mouse PrP. The RML strain used in our studies was derived from the Chandler strain, as was 139A.

Even though inocula from very different strains convert transiently expressed MHM2PrP(N180Q, N196Q) to protease-resistant MHM2PrP^{Sc}(N180Q, N196Q) equally well, the question remains whether characteristics such as the neuronal cell loss, astrocytic gliosis, PrP amyloid plaque deposition, and vacuolation patterns in brain as well as incubation times are preserved within the conformation of protease-resistant, unglycosylated PrP^{Sc}(N180Q, N196Q). Experiments with prion strains propagated in mice expressing PrP(N180Q, N196Q) transgenes may provide reliable answers to such questions. It is notable that characterization of prion strains passaged in cultured cells requires at least two passages in mice since the titres of prions in cultured cells are usually low (Butler *et al.*, 1988) and this results in a prolonged incubation time. Only on second passage in mice can a reliable incubation time for a particular strain be established.

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