Recombinant human prion protein mutants huPrP D178N/M129 (FFI) and huPrP+9OR (fCJD) reveal proteinase K resistance

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Summary
The Semliki-Forest virus (SFV) system was used to overexpress human wild-type and mutant prion proteins as well as FLAG-tagged human and bovine PrP in mammalian cells. The application of recombinant SFV vectors allowed a high-level production of highly glycosylated prion proteins with a molecular weight ranging from 25 to 30 kDa for recombinant wild-type human PrP and from 26 to 32 kDa for wild-type bovine PrP. Further, we report here the generation of recombinant mutant prion proteins that are associated with inherited human prion diseases such as fatal familial insomnia (FFI) and Creutzfeldt-Jakob disease (CJD). Both mutated variants, the FFI-associated PrP carrying a mutation at amino acid position 178 and the CJD-linked form containing an insertion of nine additional octarepeats reveal proteinase K resistance, one of the typical biochemical properties of the infectious scrapie isoform of the prion protein. By contrast, recombinant wild-type PrP was completely proteinase K sensitive when expressed in SFV-transfected BHK cells. The subcellular location of both PrP mutants at the cell surface and in intracellular compartments of transfected BHK cells was similar to that of wild-type PrP. In order to purify recombinant human and bovine PrP from cell lysates, a FLAG-tag was introduced either at the N-terminus behind the signal peptide or at the C-terminus close to the adhesion site of the GPI anchor. N-terminal insertion did not extensively influence the trafficking of the FLAG-tagged protein to the cell surface, whereas insertion close to the GPI attachment site clearly affected the transport of the majority of PrP to the cell membrane, probably resulting in their retention within the secretory pathway. All FLAG-tagged prion proteins were expressed efficiently in BHK cells and showed a typical glycosylation pattern, allowing their rapid and simple purification via anti-FLAG antibody chromatography.

Key words: Prion protein, PrP mutants, Proteinase K resistance, Semliki-Forest virus (SFV) system, Processing, Glycosylation

Introduction
The causative agent of transmissible spongiform encephalopathies (TSEs) is a prion, which is defined as a proteinaceous infectious particle (Prusiner, 1982). Prion diseases (for reviews, see Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann and Aguzzi, 1997) belong to a new class of fatal neurodegenerative disorders affecting humans and animals. The key event in the development of the disease is a conformational change in the cellular prion protein, PrPC. The misfolded, disease-associated form is termed PrPCSc, a partially proteinase-K-resistant isoform with a high β-sheet content compared with the proteinase-K-sensitive and predominantly α-helical PrPC (Caughey et al., 1991). TSEs may occur sporadically or can be inherited or transmitted. Human prion diseases include kuru, Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker syndrome (GSS) (for reviews, see Lasmézas and Weiss, 2000; Prusiner, 1998; Weissmann and Aguzzi, 1997). Whereas 85% of all CJD cases occur sporadically in the absence of any mutations, FFI, GSS and the remaining 15% of CJD are dominantly inherited diseases caused by defined mutations within the Prn-p gene on chromosome 20 (Masters et al., 1981; Sparkes et al., 1986). Genetic linkage or association has been identified for several amino-acid substitutions in the C-terminus of PrP as well as for octarepeat insertions within the N-terminal part of the molecule. Here, the spontaneous conversion of PrPC to PrPCSc in the absence of exogenous prions has been suggested (Young, 1999), but the exact mechanism by which these mutations provoke conformational changes is still unknown. Destabilization of the PrPC structure or effects on the thermodynamic stability of PrP have been suggested (Liemann and Glockshuber, 1999; Riek et al., 1998; Swietnicki et al., 1998; Zhang et al., 2000).

The physiological function of PrP is unclear. It has been proposed that PrP plays a role in synaptic processes (Collinge et al., 1994), in the regulation of circadian activity rhythms and sleep (Tobler et al., 1996) and in copper transport (Hornshaw et al., 1995). Later, a function in the survival of purkinje cells (Sakaguchi et al., 1996) was suggested, but very recently it has been reported that upregulation of the
prion-like protein doppel causes Purkinje cell degeneration in Prnp<sup>0/0</sup> mice instead of PrP depletion (Moore et al., 1999). More recently, a superoxide dismutase activity (Brown et al., 1999) and a role in signal transduction (Mouillet-Richard, 2000) for PrP have been suggested. Owing to the absence of any phenotype for PrP described in various reports (Bueler et al., 1992; Lledo et al., 1996; Manson et al., 1994), the only confirmed role of PrP<sub>c</sub> is in development of TSEs, which it appears to be essential for (Bueler et al., 1993). Cellular PrP is synthesized in the rough endoplasmic reticulum (rER) and is transported via the Golgi and secretory granules to the surface of neuronal cells where it is anchored to the plasma membrane by its glycosyl phosphatidylinositol (GPI) moiety (Rogers et al., 1991). Three different PrP glycoforms differing in their glycosylation degree have been observed on the cell surface: diglycosylated (70%), monoglycosylated (25%) and unglycosylated PrP (5%) (Caughey et al., 1989; Monari et al., 1994; Petersen et al., 1996). Recently, we identified the 37 kDa laminin receptor precursor (LRP) as an interactor for the prion protein (Rieger et al., 1997) (for reviews, see Gauczynski et al., 2001a; Rieger et al., 1999). Cell-binding and internalization studies on neuronal and non-neuronal cells have demonstrated that the 37 kDa/67 kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein (Gauczynski et al., 2001b). Direct and heparan sulfate proteoglycan (HSPG)-dependent interaction sites mediating the binding of cellular PrP to its receptor have been identified (Hundt et al., 2001). Additional heparan sulfate (HS)-binding domains in PrP<sub>c</sub> have been described (Warner et al., 2002). Cell culture experiments demonstrated the 37 kDa/67 kDa laminin-receptor-dependent binding and internalization of recombinant GST::human PrP generated in insect cells and glycosylated human PrP synthesized in BHK cells transfected with recombinant SFVRNA (Gauczynski et al., 2001b).

High-level expression and purification of recombinant, glycosylated prion proteins in mammalian cells are essential for a better understanding of the physiological function of PrP<sub>c</sub> and biochemical processes responsible for familial prion diseases. The synthesis and study of wild-type as well as mutant PrP in cell culture systems allows a better insight into the biology of these proteins, owing to the presence of important organelles, membranes and other cellular co-factors that are necessary for the correct processing, trafficking and localization of the protein. Therefore, we used the Semliki-Forest virus (SFV) system to express high amounts of glycosylated wild-type and mutant disease-associated prion protein in cultured mammalian cells. The SFV system supplies a multitude of advantages for the expression of recombinant proteins in mammalian cells: (i) large-scale production for up to 72 hours post-transfection; (ii) a broad host range; (iii) modifications such as glycosylation in a correct and sufficient way; and (iv) an easy and fast transfection procedure with in-vitro-transcribed RNA. SFV is an insect-borne alphavirus and belongs to the family of Togaviridae (Schlesinger, 1986). Its viral genome consists of capped and polyadenylated single-stranded RNA of positive polarity and encodes its own RNA polymerase. SFV expression vectors are based on a cDNA copy of the viral genome. Here, viral structural genes are deleted and replaced by the gene of interest. Owing to the remaining viral replicase, which leads to an efficient production of recombinant RNA within the cell, a high-level synthesis of the foreign protein occurs (Liljestrom and Garoff, 1991).

In the work described here, we have generated recombinant disease-related mutant isoforms of human PrP in BHK cells transfected with recombinant SFV RNAs. The FFI-associated mutant PrP D178N/M129 and the CJD-related PrP<sub>1-253</sub> were efficiently and highly glycosylated when expressed in cultured cells. We further examined biochemical features, such as the glycosylation status and proteinase K resistance of recombinant mutated PrP in comparison to recombinant wild-type PrP, as well as subcellular localization in transfected BHK cells. We observed that both mutants were proteinase K resistant at 8 μg/ml and share this biochemical hallmark with infectious PrP<sub>c</sub>. Therefore, the SFV system acting as a powerful expression system for high-level production of glycosylated prion proteins also functions as an appropriate cell culture model for inherited human prion diseases.

To facilitate purification and to introduce an additional epitope for immunodetection, we introduced a FLAG-tag at different positions in the cellular human and bovine PrP. FLAG-tag insertions at the C-terminus of PrP were located two amino acids nearer to the N-terminus than the GPI-anchor site and allowed the purification of predominantly diglycosylated human and bovine PrP from cell lysates by anti-FLAG antibody affinity chromatography.

Materials and Methods
Cell culture
Baby hamster kidney cells (BHK-21 C13; ATCC CCL 10) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO<sub>2</sub>.

Recombinant pSFV plasmid constructions
pSFV1 (Liljestrom and Garoff, 1991), pSFV3-lacZ (Life Technologies) and the ORFs from human PrP and bovine PrP were used. The plasmid DNAs pSFV1-boPrP1-264, pSFV1-huPrP1-253 (Krasemann et al., 1996), pSFV1-huPrP-FFI (encoding aa 1-253 of the human PrP containing a FFI-associated mutation at codon 178 Asp→Asn plus Met at position 129) and pSFV1-huPrP+9OR (encoding aa 1-253 of the human PrP including nine additional octapeptide repeats) (Krasemann et al., 1995) were used. pSFV1-huPrP222FLAG was generated by the QuikChange<sup>™</sup> site-directed mutagenesis method (Stratagene) employing pSFV1-huPrP1-253 DNA as a template. Construction of pSFV1-huPrP222FLAG (insertion of the FLAG-tag encoding sequence between codons 227 and 228 of the human PrP sequence) was described elsewhere (Hundt et al., 2002).

Construction of pSFV1-boPrP239FLAG
The insertion of a FLAG-tag-encoding sequence between codons 239 and 240 of the bovine PrP sequence was performed by PCR using the pSFV1-boPrP1-264 plasmid DNA as a template. A 125 bp fragment (with the FLAG-encoding sequence inserted) that encodes the C-terminus of boPrP was amplified, introducing a HindI restriction site (at codons 232-233 of the endogenous sequence) at the 5′ end, the tag-encoding sequence between codons 239 and 240 as well as a Smal site at the 3′ end. This PCR fragment encoding the C-terminal part of boPrP was digested with HindI and Smal and ligated via the HindI restriction site to a 707 bp fragment encoding the N-terminal part of boPrP from pSFV1-boPrP1-264 digested with HindI and Smal. The ligated DNA
fragments were cloned into the expression plasmid pSFV1 via the Smal restriction sites resulting in pSFV1-boPrP1-239FLAG240-264. All constructs were confirmed by dyeideo sequencing.

**SFV-mRNA generation by in vitro transcriptions**

DNAs pSFV3-lacZ (Life Technologies), pSFV1-huPrP1-253, pSFV1-huPrF1-FFL, pSFV1-huPrP+9OR, pSFV1-huPrP227FLAG, pSFV1-huPrP1-264 and pSFV1-boPrP239FLAG were linearized with SpeI following purification by phenol-chloroform extraction. Transcripts were carried out in a total volume of 50 μl containing 1.5 μg linearized plasmid DNA, 10x SP6 transcription buffer (0.4 M Tris-HCl, pH 8.0 at 20°C; 60 mM MgCl₂; 100 mM dithiothreitol; 20 mM spermidine), 1 μM of each ATP, CTP and UTP, 500 μM of GTP, 1 μM of m7G(5')ppp(5')G, 50 units of RNasin and 50 units of SP6 RNA polymerase and incubated for 2 hours at 37°C. The correct length of the transcripts was verified by agarose gel electrophoresis. RNA was stored at –20°C.

**Cell transfection**

For transfection, BHK cells were trypsinized, washed once in PBS (w/o MgCl₂ and CaCl₂) and resuspended in PBS, resulting in 1x10⁷ cells per ml. 800 μl of the cell suspension was mixed with the individual SFV-RNA and then transferred to a 0.4 cm cuvette. Transfections were carried out by electroporation at room temperature and heating at 95°C for 5 minutes. Deglycosylation was monitored by SDS-PAGE followed by western blotting and detection with the monoclonal anti-PrP antibodies 3B5 or the recombinant SFV-boPrP239FLAG RNA was diluted in complete growth medium and plated for 2 hours at 37°C. The correct length of the transcripts was verified by agarose gel electrophoresis. RNA was stored at –20°C.

**Deglycosylation assays**

8x10⁶ BHK cells were transfected with recombinant SFV RNA as described above and plated on 10 cm culture dishes. 24 hours post-transfection, cells were washed once with PBS, scraped off in PBS, harvested by centrifugation and finally lysed in 250 μl N-glycosidase F buffer by repeated freezing and thawing. The crude lysates were obtained by centrifugation at 20,200 g 4°C for 15 minutes. A 20 μl aliquot of the supernatants was then treated with 2 units of N-glycosidase F (Roche Diagnostics) at 37°C over night. In addition, aliquots of 50 μl of the supernatants were incubated with or without 0.5 units/ml endoglycosidase H (Roche Diagnostics) at 37°C for 3 hours. The reactions were stopped by addition of SDS-loading buffer and heating at 95°C for 5 minutes. Deglycosylation was monitored by SDS-PAGE followed by western blotting and detection with the monoclonal anti-PrP antibody 3B5.

**Proteinase K digestion**

8x10⁶ BHK cells were transfected with recombinant SFV RNA as described above and plated on 10 cm culture dishes. 24 hours post transfection cells were washed once with PBS, scraped of in PBS and harvested by centrifugation. Cells were resuspended in lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% Triton-X100, 0.5% DOC) and finally lysed on ice for 15 minutes. The crude lysates were obtained by centrifugation at 20,200 g 4°C for 15 minutes. Proteinase K (Roche Diagnostics) was added to a 20 μl aliquot to get the final concentrations of 2, 4 and 8 μg/ml. Reactions were carried out at 37°C for 30 minutes, stopped with 0.5 mM phenol and analysed by western blotting using the anti-PrP mAb 3F4. Protein concentrations were determined using an ELISA reader.

**Western blot analysis**

After addition of Laemmli buffer, protein samples were separated on 12% SDS-polyacrylamide gels by SDS-PAGE and transferred to PVDF membranes Immobilon P (Millipore Corp.) for 1.5 hours at 55 V. The membranes were blocked with 1-Block (Tropix) in Tris-buffered saline pH 7.5 supplemented with 0.05% Tween, probed with one of the monoclonal anti-PrP antibodies (3B5; 3F4) or the polyclonal anti-LRP antibody W3 and thereafter with an appropriate peroxidase-coupled secondary antibody. The immunoreactivity was visualized by enhanced chemiluminescence (NEN™ Life Science Products) on Kodak BioMax MR-1 films and by staining with diaminobenzidine-tetrahydrochloride (Sigma).

**Immunofluorescence analysis**

To investigate the cellular localization of recombinant prion proteins in BHK cells, we used immunofluorescence microscopy. 24 hours post-transfection, cells were washed three times with PBS and fixed with 4% paraformaldehyde. Non-permeabilized cells were fixed with 2% paraformaldehyde. After rinsing three times with PBS, cells were permeabilized for cytoplasmic staining with 0.2% Triton X-100 (10 minutes for 4°C). The preparation was saturated with a 10% FCS solution (in PBS) for 1 hour at room temperature, washed and incubated with the primary antibody mAb 3B5 diluted in PBS with 10% FCS for 1 hour at room temperature. After washing three times with PBS, the preparations were diluted in saturation buffer and incubated in the dark for 45-60 minutes with the secondary antibody (goat anti mouse) conjugated with indocarbocyanine (Cy3) (red). For nuclear staining 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature was used. Please note that both the primary and secondary antibodies were added after fixing the cells. The coverslip was mounted with aqueous mounting medium (Fluoromount®), and the slides were examined using an axioviert fluorescence microscope (Zeiss) with appropriate filters. Immuno fluorescence images were processed using Metamorph software®.

**Purification of FLAG-tagged prion proteins by anti-FLAG antibody chromatography**

Transfection of BHK cells with the recombinant SFV-huPrP227FLAG or the recombinant SFV-boPrP239FLAG RNA was performed as described above. The total volume of the electroporated cells was plated on 10 cm dishes containing 15 ml of complete growth medium followed by incubation for at least 48 hours at 37°C. 48 hours post-transfection, cells were harvested, washed once with PBS and then lysed in PBS supplemented with 0.1% Triton-X100 by repeated freezing and thawing. The crude lysates were obtained by centrifugation at 20,200 g 4°C for 15 minutes. The FLAG-tagged proteins from the supernatants were bound over night to anti-FLAG M2 affinity gel (Sigma) by rotating at 4°C. Beads with immobilized protein were then washed four times with TBS, eluted overnight by competition with 1 ml TBS containing 100 μg/ml FLAG peptides (Sigma) and dialyzed against 20 mM HEPES, pH 7.4. The purity and the concentration of the proteins were checked by SDS polyacrylamide gel electrophoresis followed by silver staining of the gel.

**Antibodies**

For western blot analyses, monoclonal anti-PrP antibodies 3F4 (Chemicon) or 3B5 (G. Hunsmann) and the polyclonal anti-LRP antibody W3 (Gauczynski et al., 2001b) were used. For immunofluorescence analyses, mAb 3B5 and a secondary Cy3 (indocarbocyanine)-conjugated antibody (used at 1:200 dilutions) (Jackson Laboratories and Southern Biotechnology, respectively) were used.
Results
Expression of glycosylated wild-type and mutant prion proteins in BHK cells transfected with recombinant Semliki-Forest virus RNAs

In order to produce high amounts of recombinant prion proteins in mammalian cells, we used the SFV expression system, which is based on a cDNA copy of the viral replicon. The transfection efficiency of BHK cells used throughout all our experiments was almost 100% (data not shown), warranting the high-level production of glycosylated prion proteins. BHK cells were transiently transfected with recombinant SFV RNAs encoding full-length human PrP encompassing amino acids 1 to 253, human PrP containing the FFI-associated mutation at codon 178 (D178N,129M), human PrP with the CJD-linked insertion of nine octarepeats and wild-type bovine PrP from amino acids 1 to 264. Western blot analysis of crude lysates demonstrates the expression of di-, mono- and low amounts of non-glycosylated forms of PrP, with a molecular weight ranging from 25 to 30 kDa for wild-type and FFI-associated human PrP and from 31 to 37 kDa for huPrP+9OR and from 26 to 32 for bovine PrP (Fig. 1, lanes 1, 3, 5 and 7), respectively. Also high molecular weight, that is hyper-glycosylated forms, of cellular and mutant PrP were synthesized and detected by the mAb anti-PrP 3B5 (Fig. 1). To confirm the glycosylation state of recombinant prion proteins, cell lysates were treated with N-glycosidase F, leading to a decrease in di- and monoglycosylated forms accompanied by an augmentation of the non-glycosylated PrP (Fig. 1A, lanes 2, 4, 6 and 8). During the cellular processing of prion proteins, modifications of high mannose glycans occur within the Golgi apparatus, leading to a resistance against endoglycosidase H (Endo H). In our studies, incubation of total cell extracts with Endo H revealed a partial resistance against this enzyme, demonstrating the correct trafficking of most of the recombinant wild-type and mutant PrP (Fig. 1B).

Proteinase K status of recombinant cellular PrP and disease-associated PrP variants

The partial resistance to proteinase K is a major hallmark of PrPSc. By contrast, cellular PrP is completely proteinase K sensitive. To investigate whether recombinant FFI- and CJD-associated PrP isoforms synthesized in SFV-transfected BHK cells share PrPSc-like properties, proteinase K was added to total cell extracts at increasing concentrations of 2, 4 and 8 µg/ml followed by incubation at 37°C for 30 minutes.
Proteinase resistance of PrP mutants (Fig. 2, upper panel). Both mutant PrPs, the FFI-associated PrP as well as the CJD-related insertion mutant, were resistant towards proteinase K amounts up to 8 μg/ml, yielding proteinase-K-resistant fragments with molecular weights ranging from 23 to 25 kDa (Fig. 2, lanes 8 and 12). By contrast, wild-type PrP was completely digested after treatment with 8 μg/ml proteinase K (Fig. 2, lane 4). Comparable signals for the 37 kDa-LRP (in the absence of proteinase K) revealed that equal amounts of protein have been loaded (Fig. 2, bottom panel). Protein concentrations in the samples digested with proteinase K were adjusted.

Cellular localization of recombinant prion proteins in transfected BHK cells

In order to prove whether or not recombinant prion proteins, either wild-type or mutant, are transported to the surface of transfected BHK cells, non-permeabilized cells (Fig. 3, left panels) were analyzed by immunofluorescence (IF) microscopy. In cells, wild-type human or bovine PrP traffics to the cell surface where it appears partially in a punctuated manner (Fig. 3B,E). Virtually all cells synthesizing PrP+9OR (Fig. 3C) or PrP-FFI (Fig. 3D) showed similar staining patterns and comparable expression levels on the cell surface, suggesting that mutated PrP variants are correctly processed to the cell surface. To visualize PrP in the interior of transfected cells, cells were permeabilized prior to immunostaining. Expression of wild-type human and bovine PrP (Fig. 3B,E, right panels) as well as of both mutated PrPs (Fig. 3C,D, right panels) revealed a wide-spread staining pattern including areas surrounding the nucleus and regions of the cytoplasm. Thus, regarding the subcellular localization, no differences between PrP mutants and the cellular forms were detectable. Cells transfected with SFV RNA encoding the viral replicase showed no detectable PrP staining on either the cell surface or in the interior of permeabilized cells (Fig. 3A), suggesting that BHK cells express no or only marginal amounts of endogenous PrPs.

Expression of glycosylated FLAG-tagged prion protein

To facilitate purification of human and bovine prion proteins and to introduce an additional epitope for immuno-detection, we inserted a FLAG-tag at the C-terminus near the GPI-anchor adhesion site resulting in the constructs huPrP227FLAG and boPrP239FLAG and at the N-terminus behind the signal peptide resulting in huPrP22FLAG (Fig. 4A). FLAG-tagged PrP isoforms expressed were highly glycosylated in transfected BHK cells (Fig. 4B, lanes 1-3 and 4C, lanes 1 and 2). To confirm the glycosylation state, samples were deglycosylated by treatment with N-glycosidase F, resulting in augmentation of the non-glycosylated PrP forms (Fig. 4B, lanes 4-6 and 4C, lanes 3 and 4). The correct processing of FLAG-tagged prion proteins to the cell surface was further analyzed by treatment with endoglycosidase H (Endo H) (Fig. 4D). A portion of the non-mutated FLAG tagged prion protein huPrP22FLAG is resistant to Endo H and therefore reaches the Golgi in which high manno sugars are modified (Fig. 4D, lane 2). By contrast, both PrP forms carrying the tag close to the GPI attachment site, the huPrP227FLAG and primarily the boPrP239FLAG, feature Endo H sensitivity, suggesting that only low amounts of the highly glycosylated proteins are properly transported via ER and Golgi to the cell surface (Fig. 4D, lanes 4 and 6).

Cellular localization of FLAG-tagged prion proteins in transfected BHK cells

To investigate whether FLAG-tagged prion proteins are localized at the surface of transfected BHK cells, non-permeabilized cells were stained with anti-PrP antibody 3B5 and analyzed by IF microscopy. As already shown in Fig. 3,
human and bovine wild-type PrP were efficiently transported to the cell surface of transfected BHK cells (Fig. 3B,E; Fig. 5B,E). Owing to the overexpression of recombinant protein in recombinant SFV RNA-transfected cells, wild-type human and bovine PrP were also detectable inside permeabilized cells (Fig. 5B,E, right panels). Permeabilization of transfected cells prior to immunostaining reveals intracellular deposits of both wild-type and FLAG-tagged prion proteins (Fig. 5, right panels). Whereas huPrP22FLAG was detectable on the cell surface at comparable levels (Fig. 5C, left panel), both prion proteins carrying the FLAG-tag insertion close to the GPI-attachment site were transported efficiently only in a minority of cells to the surface of transfected BHK cells (Fig. 5D,F), which is in agreement with the negligible Endo H resistance of these proteins (Fig. 4D), suggesting that the FLAG-tag at this position hampers trafficking of PrP to the cell surface. Indeed, most of the protein remains within the interior of the cell as shown by IF analysis on permeabilized cells (Fig. 5D,F, right panels). BHK cells transfected with SFV RNA encoding for the viral replicase showed neither surface nor intracellular staining (Fig. 5A), confirming that no or only a negligible amount of cellular PrP is synthesized by BHK cells.

### Purification of glycosylated FLAG-tagged PrP

Purification of FLAG-tagged prion proteins was performed by anti-FLAG antibody chromatography. FLAG-tagged proteins from cell lysates were immobilized to anti-FLAG M2-conjugated agarose beads (Fig. 6A,B, lanes 2). Silver-stained SDS gels revealed that mainly the diglycosylated PrP form was purified by this one-step purification process (Fig. 6C). Both kinds of recombinant proteins were recognized by the PrP-specific antibody 3B5 (Fig. 6A,B, lanes 3). The huPrP22FLAG could hardly be purified by either anti-FLAG M1 or M2 antibody chromatography probably due to insufficient binding of the FLAG epitope to the anti-FLAG antibody immobilized on sepharose under native conditions.
Fig. 4. Expression of glycosylated FLAG-tagged human and bovine PrP in BHK cells transfected with recombinant SFV RNAs. (A) Constructs of human PrP containing a FLAG-tag insertion either between amino acids 227 and 228 close to the GPI-anchor adhesion site or behind the N-terminal signal peptide between amino acids 22 and 23 and a bovine PrP construct with the FLAG-tag inserted between amino acids 239 and 240. (B) Expression and deglycosylation of wild-type and FLAG-tagged human PrP. (C) Expression and deglycosylation of wild-type and FLAG-tagged bovine PrP. Total cell extracts from SFV-transfected BHK cells were incubated overnight with N-glycosidase F at 37°C and analyzed by western blotting. For detection, mAb anti-PrP 3B5 was used. FLAG-tagged prion proteins were glycosylated in the same manner as wild-type PrP (B, lane 2 and 3 and C, lane 2). After treatment with N-glycosidase F, the glycosylated forms are clearly reduced, accompanied by an increase in the non-glycosylated forms (B, lane 4, 5 and 6; C, lane 3 and 4). The shift in the molecular weight of non-glycosylated FLAG-tagged PrPs is due to the additional eight amino acids of the FLAG-tag. (D) Effects of endoglycosidase H on FLAG-tagged human and bovine PrP. Total cell extracts were incubated for 3 hours with Endo H at 37°C and analyzed by western blotting using mAb 3B5. Endo H sensitivity is shown by an increase of the non-glycosylated PrP form (D, lanes 2, 4 and 6).
Discussion

Synthesis of wild-type and mutant prion proteins in mammalian cells by the Semliki-Forest virus system

In order to solve the mystery of the physiological function of the cellular prion protein, a great effort has gone into understanding the biology and structure of PrPc. Different expression systems have been applied to produce recombinant prion proteins in prokaryotic, insect, yeast or mammalian cells. Expression of Syrian golden hamster prion protein in *E. coli* and in Baculovirus-infected insect cells using the glutathione S-transferase (GST) fusion system have been described previously (Volkel et al., 1998; Weiss et al., 1996; Weiss et al., 1995). Although these systems resulted in large-scale protein production, only unglycosylated or marginal glycosylated PrP has been obtained. Thus, we decided to establish the SFV system for high-level expression of different wild-type and mutant prion proteins in mammalian cells. As expected, recombinant glycosylated PrP was synthesized in BHK cells transfected with recombinant SFV RNAs. The typical glycosylation pattern consisting of unglycosylated, mono- and diglycosylated PrP was observed when we investigated the total cell extracts by western blot analysis. The presence of N-linked oligosaccharide chains was confirmed by N-glycosidase F treatment, which led to deglycosylation of PrP, resulting in the generation mainly of the non-glycosylated PrP form.

Processing and trafficking of wild-type and mutated human prion proteins

Correctly processed PrP traffics to the cell surface where it is anchored to the plasma membrane by its GPI moiety (Rogers et al., 1991). We investigated the subcellular location of recombinant wild-type and disease-associated mutant PrP by IF microscopy. Very recently, it has been observed that mutant PrP molecules linked to familial prion diseases partially are retained in the ER (Ivanova et al., 2001). This led to the conclusion that several pathogenic mutations, that is, point and insertion mutations within the *Prnp* gene, exert influence on the trafficking of PrP and that protein quality control might play an important role in TSEs (Ivanova et al., 2001) (for summary see Table 1). For several mutations an additional mode of membrane association resulting in resistance to enzymatic cleavage of the GPI-anchor was reported (Lehmann and Harris, 1995;
| Human PrP mutants + human TSE-associated homologues | Human disease | Expression system | Cellular localization | Proteinase K status |
|--------------------------------------------------|--------------|-------------------|-----------------------|--------------------|
| MoPrP PG14 (+9OR)                                 | fCJD         | BHK cells transfected with Sindbis viral replicon\(a\) | Low level cell-surface expression\(a\); retention in the endoplasmic reticulum\(b\) | Resistant (up to 1 \(\mu\)g/ml proteinase K for 30 minutes 37°C)\(a\) |
| MoPrP PG11 (+6OR)                                 | fCJD         | Stably transfected CHO cells (pBC12/CMV) \(c,d\) | Cell-surface expression with additional mode of membrane association\(b,c\) | Resistant (3.3 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(b,d\) |
| MoPrP E199K \(\text{bc,d}\)                      | fCJD         | Stably transfected CHO cells (pBC12/CMV) \(b,d\) | Cell-surface expression with additional mode of membrane association\(b\) | Resistant (3.3 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(b;d\) |
| MoPrP D177N/M128 \(\text{bc}\)                   | FFI          | Stably transfected CHO cells (pBC12/CMV) \(b\) | Low level cell-surface expression\(a\); additional mode of membrane association\(b\); retention in the endoplasmic reticulum\(a\) | Resistant (3.3 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(b\) |
| MoPrP P101L \(\text{bc}\)                        | GSS          | Stably transfected CHO cells (pBC12/CMV) \(b\) | Low level cell-surface expression\(a\); additional mode of membrane association\(b\); retention in the endoplasmic reticulum\(a\) | Resistant (3.3 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(b\) |
| HaPrP+24/60R \(\text{e}\)                        | fCJD         | Mouse fibroblast cells transfected with retroviral vector\(e\) | Aberrant cell-surface expression\(e\) | Resistant (up to 0.8 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(f\) |
| HaPrP+4/60R \(\text{e}\)                         | fCJD         | Mouse neuroblastoma cells transfected with retroviral vector\(e\) | Normal cell-surface expression\(e\) | Resistant (up to 1 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(f\) |
| HuPrP D178N/V129 \(\text{f}\)                   | fCJD         | Human neuroblastoma cells transfected with episomal vector\(f\) | Impaired transport of unglycosylated PrP to the cell surface\(f\) | Sensitive (at least 0.5 \(\mu\)g/ml PK 1 hour or 5 \(\mu\)g/ml proteinase K 5 minutes 37°C)\(f\) |
| HuPrP E200K \(\text{b}\)                        | fCJD         | Human neuroblastoma cells transfected with episomal vector\(b\) | Under-representation of unglycosylated PrP at the cell surface\(b\) | Resistant (3.3 \(\mu\)g/ml proteinase K 10 minutes 37°C)\(b\) |
| HuPrP D178N/M129 \(\text{f}\)                   | FFI          | Human neuroblastoma cells transfected with episomal vector\(f\) | Impaired transport of unglycosylated PrP to the cell surface\(f\) | Sensitive (at least 0.5 \(\mu\)g/ml PK 1 hour or 5 \(\mu\)g/ml proteinase K 5 minutes 37°C)\(f\) |
| HuPrP Q217R/V129 \(\text{g}\)                   | GSS          | Human neuroblastoma cells transfected with episomal vector\(g\) | Impaired transport to the cell surface\(g\); accumulation of aggregated PrP in intracellular compartments\(g\) | Resistant (3.3 \(\mu\)g/ml proteinase K 5 minutes 37°C)\(g\) |
| HuPrP+9OR                                        | fCJD         | BHK cells transfected with Semliki Forest virus RNA | Expression at the cell surface and in intracellular compartments | Sensitive* (8 \(\mu\)g/ml proteinase K 30 minutes 37°C) |
| HuPrP D178N/M129 \(\text{f}\)                   | FFI          | BHK cells transfected with Semliki Forest virus RNA | Expression at the cell surface and in intracellular compartments | Resistant* (8 \(\mu\)g/ml proteinase K 30 minutes 37°C) |

fCJD: familial Creutzfeldt-Jakob disease; FFI: fatal familial insomnia; GSS: Gerstmann-Sträussler-Scheinker syndrome.
MoPrP: mouse PrP; HaPrP: hamster PrP; HuPrP: human PrP; +OR: additional octarepeats.
\(a\)Ivanova et al., 2001; \(b\)Lehmann and Harris, 1996a; \(c\)Lehmann and Harris, 1995; \(d\)Lehmann and Harris, 1996b; \(e\)Priola and Chesebro, 1998; \(f\)Petersen et al., 1996; \(g\)Singh et al., 1997; \(h\)Capellari et al., 2000.

*Data described in this manuscript.
Lehmann and Harris, 1996a), a phenomenon that is also typical for infectious PrPSc. Previous studies have already shown that mutant prion proteins are inefficiently processed to the cell surface and accumulate in intracellular compartments such as the ER, Golgi and endosomes/lysosomes (Capellari et al., 2000; Jin et al., 2000; Petersen et al., 1996; Singh et al., 1997; Zanusso et al., 1999) (for summary see Table 1). By contrast, IF analysis of non-permeabilized, transfected BHK cells expressing huPrP-FFI or huPrP+9OR revealed similar surface staining in comparison to cells expressing wild-type human PrP at their cell surface. In addition, the intracellular staining of wild-type as well as both mutant PrP isoforms revealed the same wide-spread pattern encompassing nearly the entire interior of the cell. This effect might be caused by the SFV system, owing to the hyperexpression of recombinant protein while competing out the host translation machinery. Therefore, it might be difficult to detect subcellular, trapped PrP in cells overexpressing this protein. However, we cannot exclude the possibility that mutant PrP molecules are partially retained in cellular compartments such as the ER. Endoglycosidase H (Endo H) digestions, however, revealed that the FFI- and CJD-associated PrP mutants are partially Endo H resistant, suggesting that high mannose sugars have been added to most of the recombinant prion proteins followed by modifications on their way through the ER and the Golgi apparatus to the cell surface. This finding is in contrast with the hypothesis that the mutant prion proteins might be trapped in compartments of the secretory pathway and instead confirms the finding obtained by IF analyses (Fig. 3) that mutant prion proteins are located at the cell surface.

Proteinase K resistance of mutated human prion proteins
One major biochemical characteristic of PrPSc is its resistance to proteinase K. Some mutant isoforms of the mouse prion protein carrying mutations homologous to familial human prion diseases were produced in stably transfected CHO cells and displayed scrapie-like biochemical properties such as proteinase K resistance (for summary see Table 1) and detergent insolubility (Daude et al., 1997; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b). Furthermore, hamster insertion mutants encompassing additional octarepeats showed protease resistance when synthesized in cultured cells (Table 1) (Priola and Chesebro, 1998). To investigate whether genetically linked human PrP mutants expressed in transiently transfected BHK cells behave in a similar manner to PrPSc, total cell extracts were treated with proteinase K at final concentrations of 2, 4 and 8 μg/ml for 30 minutes at 37°C. Under these conditions, we detected a protease-resistant fragment of FFI-associated and CJD-related human PrP with a molecular weight ranging from 23 to 25 kDa. Previous reports describing partial PK resistance for disease-associated PrP used lower proteinase K concentrations and/or shorter incubation times (for a summary, see Table 1). The mouse PG14 PrP mutant encompassing nine additional octarepeats was proteinase K sensitive at a concentration of 2 μg/ml when synthesized in stably transfected BHK cells using the Sindbis replicon (Ivanova et al., 2001). In addition, the human PrP mutant D178N/M129 was totally degraded when digested with low amounts of PK for 1 hour or with 5 μg/ml PK for at least 5 minutes (Table 1). It is difficult to compare the characteristics of mutant PrPs of different species, expression systems and cells and the diverse conditions of proteinase K digestion. Employing the SFV system, we were able to synthesize glycosylated, cell-surface orientated and proteinase-K-resistant (up to 8 μg/ml) human PrP mutants, recommending its capability as a cellular model system for familial human prion diseases. Bioassays will prove whether these PK-resistant PrP molecules will harbor endogeneous infectivity.
Processing, trafficking and purification of FLAG-tagged human and bovine prion proteins

To purify prion proteins rapidly and simply from total cell extracts and to introduce an epitope suitable for specific immunogenic detection, a FLAG-tag was inserted within the human PrP-encoding sequence close to the GPI anchorage site at residue 227 or at the N-terminus following the signal peptide sequence at residue 22. A homologous FLAG-tag insertion within the bovine PrP sequence termed boPrP239FLAG was also constructed. The locations of the FLAG-tag insertion consisting of the eight amino-acid sequence Asp-Tyr-Lys-Asp-Asp-Asp-Lys, influenced the processing of bovine and human PrP\(^\text{c}\) in transfected BHK cells. HuPrP22FLAG was processed normally and expressed at high levels similar to wild-type PrP on the cell surface of transfected cells. By contrast, introduction of the FLAG-tag close to the GPI-attachment site hampered the transport of huPrP227FLAG and boPrP239FLAG to the cell surface. Only low amounts of both proteins reached the cell surface. One possible explanation for this phenomenon might be the hampering effect of the FLAG-tag, which was inserted close to the GPI attachment site, leading to a disruption of the transit of huPrP227FLAG and boPrP239FLAG to the cell surface and a retention of these proteins within cellular compartments of the secretory pathway such as the ER. This explanation is supported by the finding that huPrP227FLAG and boPrP239FLAG are both sensitive towards Endo H digestion, whereas huPrP22FLAG is more Endo H resistant. Recombinant glycosylated huPrP227FLAG and boPrP239FLAG have been purified from crude lysates by anti-FLAG-antibody chromatography. Such an epitope-tagged prion protein, which can be purified simply and rapidly from total cell extracts might represent a useful tool to elucidate the physiological function of PrP\(^\text{c}\) and its role in the pathological mechanisms of TSEs.

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