Cellular Phenotyping of Secretory and Nuclear Prion Proteins Associated with Inherited Prion Diseases*

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The pathogenic mechanisms leading from mutations in the prion protein (PrP) gene to infectious disease are not understood. To investigate the possibility that cellular processing of mutant prion protein may contribute to the formation of infectious particles, a mouse PrP model system has been established using the green fluorescent protein. Three novel PrP mutants were examined employing this model system and compared with wild type as well as known mutant PrPs. Two Creutzfeldt-Jakob disease-associated PrP mutants, PrP T188K and PrP T188R, revealed a secretory pathway to the cell membrane and PrPSc-like properties, i.e. enhanced protease K resistance and detergent insolubility similar to other mutant PrPs associated with familial prion diseases. Moreover, a recently described disease-related truncated PrP mutant, PrP Q160Stop, showed an almost exclusive localization in the nucleus and a catabolism along the proteasomal pathway. Therefore, various distinct pathological mechanisms may cause prion diseases, and aberrant cellular processing may be included in the pathogenesis of prion diseases.

Transmissible spongiform encephalopathies in animals and man, also known as prion diseases, are rare fatal neurodegenerative disorders that can occur in sporadic, acquired, or autosomal-dominant heritable forms (1). All inherited prion diseases have been found to segregate with mutations in the coding region of the human prion protein gene (PRNP) on the short arm of chromosome 20. So far, more than 20 mutations have been detected, which are causative for prion diseases, including insertions and point and nonsense mutations. Classically, three heritable forms are distinguished according to their clinicopathological presentation, i.e. familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia. These diseases are unique in that they can be inherited as well as transmitted to laboratory animals (via an infectious process), implying that infectious agents, called prions, are formed de novo in affected individuals. Prions consist, at least to a large extent, of a conformationally altered isoform (PrPSc) of a host-encoded glycoprotein, PrPSc. Biochemically, several differences have been described between PrPSc and PrPC, the most prominent being the enhanced protease K (PK) resistance of PrPSc and its insolubility in non-denaturing detergents. The cellular function of the prion protein is still not entirely understood, but its localization on the cell membrane makes PrP a possible candidate for a cell adhesion or receptor molecule. Recently, it has been suggested that PrPSc may play a role in copper homeostasis and metabolism, since PrP binds copper via its N-terminal peptide repeat region (2–4). In addition, these data point to a function either in synaptic interaction or in the sensitivity of neurons to oxidative stress (5, 6). It remains unclear whether the disease-associated mutants of PrP exert their effects by loss-of-function or gain-of-(dys)function of the protein and whether the resulting pathogenic mechanisms are identical to those in sporadic disease. It has, however, been demonstrated in several studies employing cell culture systems that PrPSc from genetic as well as sporadic cases is very similar with respect to its biochemical characteristics (7, 8).

To examine the fate of PrP in living cells, the green fluorescent protein (GFP) was inserted as a marker into the N terminus of the mouse prion protein (9, 10), similar to a strategy recently published for bovine PrP (25). GFP chimeras with wt as well as mutant PrPs were expressed transiently under the control of a tetracycline-inducible promoter in murine neuroblastoma (N2a) cells (11). The mutant PrPs are mouse homologues of human pathogenic PrPs linked to different types of inherited prion diseases (Table I). The translocation and metabolism of the prion proteins were examined biochemically and by means of confocal laser scanning microscopy (CLSM). Thus, aberrant biochemical as well as cellular characteristics could be revealed for newly described PrP mutants, e.g. an exceptional strong nuclear localization of a shortened PrP molecule, which is rapidly degraded through the proteasomal pathway.

EXPERIMENTAL PROCEDURES

Cells, Cell Culture Conditions, and Production of Transfected Cell lines—The murine neuroblastoma cell line N2a (12) was obtained from the American Type Culture Collection (Manassas, VA). Generation of the stably transfected N2a cells (N2a-tTA, clones 26 and 29) expressing the tetracycline transactivator (tTA) has been described previously (11). All cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and penicillin/streptomycin in an atmosphere of 10% CO2, 90% air at 37 °C.

Transient transfections of N2a and N2a-tTA cells were performed using the Effectene reagent (Qiagen) according to the manufacturer’s instructions. All DNA used for transfections possessed high quality standard (Qiagen) and matched concentrations. Cells were grown for Western blot analysis directly on multi well dishes and, for confocal microscopy studies, on glass coverslips.

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**Cellular Phenotyping of Prion Proteins**

PrP Constructs—Construction of pBS-Prnp and pHCMV+-Prnp has been described previously (11). The plasmid pBS-GFP-PrP was constructed by inserting the GFP-S65T DNA sequence of vector pS65T-C1 (CLONTECH) in-frame into the Smal site of the Prp open reading frame (13) in pBS-Prnp. The 5’-end of the G65T-S65T-DNA was supplemented with an 18-bp XbaI linker to accommodate the Clontech constraints. The P/JIM- BamHI fragment of pBS-GFP-PrP was then cloned into the P/JIM-BamHI site of pHCMV+-Prp to yield pGFP-PrP. Details of plasmid constructions are available upon request. P101L (primers 1 and 2), W144(G/G) (primers 3 and 4), Q159(G/G) (primers 5 and 6), D177N (primers 7 and 8), T187K (primers 9 and 10), T187R (primers 11 and 12), and E199Q (primers 13 and 14) mutations of the mouse prion protein open reading frame were obtained by a PCR-based site-directed mutagenesis method (14) with pBS-Prnp as template using the following primers: 5’-TCAAGGACACAAAAAACG-3’ (primer 1), 5’-GCTGTGGTCCATCAGTTA-3’ (primer 2), 5’-CATTGGTGCCACAGTACTGAGGACGCTGCTACTACGG-3’ (primer 3), 5’-ATCATGCTGCCCTGTCACCGGCCTCCCACCC-3’ (primer 4), 5’-GATCCGTCTACCCATACTAGTGA-3’ (primer 5), 5’-AGTTTTCATCGTATAGCGTGCTCATCGCAG-3’ (primer 6), 5’-AAGTGCTGCAATCACTACCCACCC-3’ (primer 7), 5’-GGTCAAGCTTGGTTCG-3’ (primer 8), 5’-CATCAAGCATACAGCCTACCCACCAACG-3’ (primer 9), 5’-TGGTATAGGCGCCGCTTGC-3’ (primer 10), 5’-GGTCAACCAACCAAGGGCTA-3’ (primer 11), 5’-TGGTGCTGCTATGATGATGATGATTG-3’ (primer 12), 5’-AAGGTTGTACATTCGTCCTCCGCCTGCT-3’ (primer 13), and 5’-TCATTGTTCAATGCTCTACGTCCCTCCCTGTC-3’ (primer 14). The Bsa61RHI fragment of positively mutated Prnp in pBS-Prnp replaced the Bsa61RHI-BamHI wt Prp fragment of pGFP-PrP. Additionally, for permanent expression of mutagenesis events were confirmed by sequence analysis. pcGFP-wtPrP of normal N2a cells with pcGFP-wtPrP/H11032 (primer 12), 5’-CGCGCTCCCC-3’, yielded the same cellular phenotype of GFP-wtPrP as transfections of (Invitrogen). Transfections of normal N2a cells with pcGFP-wtPrP/H11032 yielded the same cellular phenotype of GFP-wtPrP as transfections of normal N2a cells with pcGFP-wtPrP/H11032 (primer 11), 5’-CGCGCTCCCC-3’. Transfection with p Bs-Prnp as template using the deglycosylation with peptide — glycosidase F (New England Biolabs) for 4 ha t 37 °C. The extracts were treated with 500 units of peptide N-glycosidase F for 4 h at 37 °C according to the manufacturer’s instructions and assayed by Western blot analysis.

**Assay of Metabolic Effects on PrP by Inhibition of Degradation Pathways**—Equal amounts of cell cultures were incubated for 48 h post-transfection with 1.5 mM chloroquine. The proteasome inhibitors MG-115 and lactacystin was from Sigma. Afterward, cells were lysed in TNE buffer containing 0.2% sarcosyl, and cell extracts were subjected to Western blot analysis. The cells that were maintained on the microscale stage without any gas change using a slide chamber fitted for 12-mm round coverslips together with a heating plate. For multi-channel imaging, each fluorescent dye was dyed sequentially in the frame-interlace mode to eliminate cross talk between the channels. GFP fluorescence was excited with a 488-nm argon-ion laser and imaged using a RSP 500 main beam splitter and a 515-nm long-pass filter. Alexa 594 and ceramide TR fluorescence was excited with a 543-nm HeNe laser line and imaged using a 560-nm long-pass filter settings. All image processing was performed using the Leica TCS NT software; Adobe Photoshop was used to prepare final figures for publication.

**RESULTS**

**GFP-wtPrP Is Processed and Localized Like Native PrPC**—The protein chimera GFP-wtPrP was created by the insertion of the GFP-S65T DNA sequence between codons 39 and 40 of the wt mouse PrP open reading frame (Fig. 1A). To compare the biosynthesis of the GFP-wtPrP chimera with that of wt PrPC, the expression of both was first examined biochemically. Western blot analysis of transfected recombinant N2a cells (11) confirmed the synthesis of glycosylated proteins with molecular masses of about 48–58 kDa for GFP-wtPrP and 27–37 kDa for native PrPC. Non-chimeric as well as chimeric PrP could be detected with antibodies against mouse PrP, but GFP-wtPrP could also be recognized by antibodies against GFP (Fig. 1B). To investigate whether the cellular transport of GFP-wtPrP is comparable with that of the secretory and membrane-bound protein PrPC, the localization of GFP-wtPrP in N2a cells was observed from about 20 h post-transfection by CLSM and assayed by co-localization studies. Thereby, a co-localization of GFP-wtPrP could be detected with an ER marker (Fig. 1C, upper panel) and most prominently with a Golgi marker (Fig. 1C, lower panel), but no co-localization was detectable with organelle markers for mitochondria, cytoskeleton components, or the nucleus (data not shown). Further observation of post-Golgi transport of GFP-wtPrP revealed the enrichment of fluorescent signals on the cell surface (Fig. 1D), and prolonged observations (here shown for 48 h post-transfection, but also detectable in long term, stable N2a clones expressing GFP-wtPrP) confirmed a final GFP-wtPrP signal distribution in transfected N2a cells with prominent localizations in the Golgi apparatus and on the cell membrane (Fig. 1E). To exclude the possibility that even incomplete GFP-wtPrP chimeras show an identical cellular phenotype like GFP-wtPrP, control fusion proteins were designed carrying either the PrP sequence C-terminally (GFP-PrPDN) or N-terminally (GFP-PrPAC) in addition to GFP (Fig. 1A). Both fusion proteins could be detected

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2 H. Lorenz, unpublished result.
in Western blot analysis as truncated and unglycosylated proteins (data not shown) and could furthermore be observed in confocal microscopy studies as homogeneously distributed signals throughout the cytoplasm and the nucleus, similar to the confocal microscopy studies as homogeneously distributed signals.

Expression of Chimeric PrP Mutants—To investigate the processing and transport of PrP mutants in N2a cells by this model system, seven GFP-PrP chimeras were constructed that correspond to several distinct mutations in inherited human prion disease (Table I). Among these PrP mutants, there were three, GFP-PrP Q159Stop, GFP-PrP T187K, and GFP-PrP T187R (corresponding to human PrP Q160Stop, T188K, T188R), that have so far never been cellurally examined and which were assayed in this study most intensively. In addition, four celluarily well defined PrP mutants (GFP-PrP P101L, GFP-PrP W144Stop, GFP-PrP D177N, and GFP-PrP E199K as the mouse homologues for human PrP P102L, Y145Stop, D178N, E200K) were used as controls to evaluate the GFP-PrP model system and to extend the existing knowledge about these mutants (7, 17, 19, 20). After expression in N2a cells, the chimeric PrP nonsense mutants, i.e. GFP-PrP P101L, GFP-PrP D177N, and GFP-PrP E199K, were fully glycosylated and synthesized to molecular masses of about 48–58 kDa like the chimera with wt PrP (Fig. 2A). All these glycosylated chimeras were sensitive to deglycosylation by digestion with peptide N-glycosidase F (data not shown), as is the case with native PrP. The glycosylation of the GFP-PrP chimeras is therefore neither blocked by the insertion of GFP nor by the disease-linked point mutations in mouse PrP.

In contrast to the missense mutants, the chimeric PrP missense mutants GFP-PrP P101L, GFP-PrP D177N, and GFP-PrP E199K appeared in Western blot analysis as truncated and double-banded proteins of about 40–42 and 41–43 kDa, respectively (Fig. 2A).

To test full-length chimeric PrP mutants with respect to their resistance against proteolysis, total cell extracts of transfected N2a cells were digested under mild conditions with PK.
Characteristics of chimeric PrP mutants. A, expression of chimeric GFP-PrP mutants in N2a cells. N2a cells were extracted 48 h post-transfection, and equal amounts of lysates (100 μg each) were analyzed by immunoblotting with monoclonal antibody against GFP. Lane 1, GFP-wtPrP; lanes 2–8, GFP-PrP mutants with amino acid substitutions P101L (lane 2), W144X (lane 3), Q159X (lane 4), D177N (lane 5), T187K (lane 6), T187R (lane 7), and E199K (lane 8). B, resistance of full-length GFP-PrP chimeras against proteinase K. Total protein extracts (150 μg each at equal concentrations) of N2a cells (72 h post-transfection) were treated with 5 μg/ml PK at 37 °C for 30 min and analyzed by immunoblotting with monoclonal anti-PrP antibody 6H4. Lanes 1 and 2, untreated (−) and treated (+) GFP-wtPrP; Lanes 3–7, treated GFP-PrP mutants with amino acid substitutions P101L (lane 3), D177N (lane 4), T187K (lane 5), T187R (lane 6), and E199K (lane 7). C, localization of GFP-wtPrP and chimeric mutant PrPs in N2a cells. Single confocal sections of cells expressing GFP-wtPrP, GFP-PrP P101L, GFP-PrP W144X, GFP-PrP Q159X, GFP-PrP D177N, GFP-PrP T187K, and GFP-PrP E199K (from left to right, lower panel) 48 h post-transfection. Bars, 10 μm.

Under similar conditions, enhanced PK resistance has been detected for the non-chimeric counterparts of the PrP mutants with amino acid exchanges P101L, D177N, and E199K (7, 19). Transfections were carried out under identical conditions, and 72 h post-transfection lysates were prepared and assayed with matched concentrations of total protein in equal volumes (Fig. 2B). All full-length chimeras with pathogenic mutations were tested and showed an enhanced resistance against PK leading to truncated molecules. All these resistant protein signals were clearly and reproducibly visible in Western blot analysis, indicating that the chimeric PrP mutants also underwent biochemical changes that led to increased PK resistance.

To investigate the cellular localization of the PrP mutants, the expression of the chimeras was observed by CLSM. The microscopic examination revealed that the chimeric mutant proteins GFP-PrP P101L, GFP-PrP D177N, GFP-PrP T187K, and GFP-PrP T187R showed no difference in localization from GFP-wtPrP (Fig. 2C). For all of these, fluorescent signals were detectable in the Golgi apparatus and on the cell membrane of N2a cells. These chimeras were trafficked along a secretory pathway like native PrP, and no differences in cellular transport and localization of the level of expression were detected. In contrast to the other chimeras, no fluorescent signals in the Golgi apparatus and on the cell membrane were detectable for the truncated chimeras GFP-PrP W144Stop and GFP-PrP Q159Stop (Fig. 2C). Expression of the GFP-PrP W144Stop chimera led to a distribution throughout the cytoplasm and the nucleus, whereas the GFP-PrP Q159Stop mutant showed an almost exclusive localization in the nucleus that was clearly distinguishable from GFP-wtPrP but also from GFP-PrP W144Stop. All cellular phenotypes presented here could be confirmed for the chimeric and the corresponding non-chimeric PrP mutants by immunofluorescence analysis with anti-PrP antibodies (data not shown).

Cellular Trafficking of the Chimeric PrP Mutants.—The three chimeric PrP mutant proteins GFP-PrP T187K, GFP-PrP T187R, and GFP-PrP Q159Stop were further analyzed with respect to their cellular transport. To investigate the intracellular translocation between ER and Golgi apparatus, the transfected cells were incubated with the fungal metabolite brefeldin A (BFA). BFA reversibly initiates the retrograde transport of Golgi vesicles into the ER, which can be stopped by a washout of the drug that leads to a subsequent reorganization of the Golgi apparatus (21). By using BFA as a cellular tool, secretory proteins could be cycled from the Golgi into the ER and back, which allows the determination and the microscopic observation of the cellular pathway(s) of the GFP-PrP chimeras. Time-lapse observations of the GFP-wtPrP signal distribution in BFA-treated N2a cells revealed the complete disappearance of discrete Golgi signals within 60 min and the appearance of an "ER-typical" signal distribution (Fig. 3A). This BFA-induced phenotype was also detected in N2a cells expressing GFP-PrP T187K and GFP-PrP T187R (Fig. 3B) but was never observed for GFP-PrP Q159Stop-expressing cells (Fig. 3A). Transfected cells showed redistribution of GFP-PrP T187K and GFP-PrP T187R into the ER in the presence of BFA and a reorganization of discrete fluorescent signals or Golgi signals, respectively, in the absence of BFA, which clearly indicates a secretory pathway along the ER/Golgi complex for these two chimeric PrP mutants (Fig. 3B). In contrast the chimeric PrP nonsense mutant GFP-PrP Q159Stop showed no effect on BFA incubation of the cells. The signal distribution remained restricted to the nucleus, and no changes in the signal localization occurred even after BFA incubation for up to 20 h (data not shown), indicating that GFP-PrP Q159Stop never entered the Golgi apparatus along its cellular pathway.
To investigate the localization of the chimeric PrPs on the cell surface, the transfected cells were incubated with phosphatidylinositol-specific phospholipase C (PIPLC), which cleaves GPI-anchored proteins like native PrP from the cell membrane. During PIPLC treatment for up to 40 min, the GFP-wtPrP, GFP-PrP T187K, and GFP-PrP T187R-expressing cells showed an ongoing disappearance of the fluorescent signals on the cell surface, whereas no loss of fluorescence was observed for cells expressing GFP-PrP Q159Stop (Fig. 4). To exclude that hypothetically existing membrane-bound GFP-PrP Q159Stop was for some reason not accessible to PIPLC, we applied the prototye trypsin into the culture medium and assayed the trypsin digest by Western blot analysis of whole cell extracts. However, whereas high reductions of the protein content were detected for N2a cells expressing the secretory chimeric PrPs, no effect on the protein amount was measurable for GFP-PrP Q159Stop (data not shown). This result supported the confocal recordings that GFP-PrP Q159Stop seemed to be exclusively localized in intracellular compartments without any trafficking along the ER/Golgi complex to the outer-membrane like wt and several mutant PrPs (Fig. 2C).

Expression and Cellular Trafficking of Non-chimeric PrP Mutants—Further examinations of PrP trafficking were carried out with the non-chimeric mouse wt and mutant PrPs. Corresponding to the analysis of the chimeric PrPs, the synthesis of the native PrPs was first examined biochemically. Western blotting revealed that the expression in N2a cells yielded glycosylated wt PrP, PrP T187K, and PrP T187R with molecular masses of ~27–37 kDa (Fig. 5A). Similar to the chimeric counterparts, double-banded signals could also be detected for the truncated PrP mutants, with molecular masses of ~14 and 16 kDa for PrP W144Stop and 15 and 17 kDa for PrP Q159Stop (Fig. 5A). Therefore, the expression pattern of the native PrPs clearly resembled that of their chimeric homologues and revealed the expected molecular weights of the prion proteins without the GFP portion.

To evaluate the amount of secreted PrP of the PrP mutants, transfected cells were cultured in fresh salt solution (physiological) for 45 min, and the proteins in the culture medium were methanol-precipitated and subjected to Western blot analysis. Under these conditions, secretion into the medium could easily be detected for wt PrP, PrP T187K, and PrP T187R but not for PrP W144Stop and PrP Q159Stop (Fig. 5B), supporting our data that the PrP nonsense mutants were strictly restricted to intracellular regions and, in contrast to the other examined PrPs, not trafficked along a secretory pathway. Identical results were also obtained for the corresponding chimeric PrPs (data not shown).

Biochemical Properties of the PrP Mutants—To investigate whether PrP T187K and PrP T187R also exhibit an enhanced PK resistance like many other disease-associated full-length PrP mutants (7, 8) or their chimeric PrP homologues, respectively (see Expression of Chimeric PrP Mutants in the “Results” section), whole cell extracts of N2a cells expressing PrP T187K and PrP T187R were digested with PK (5 μg/ml, 37 °C, 30 min) and assayed by Western blotting. Similar to their chimeric counterparts and in contrast to wt PrP, mutant PrP T187K and PrP T187R showed an enhanced PK resistance leading to truncated instead of totally digested protein and, by this, confirming the results of the GFP-PrP chimeras (Fig. 6A). In addition, the PK resistance of the shortened PrP mutants, W144Stop and Q159Stop, was also examined by applying identical conditions of PK incubation as used for the full-length PrP mutants. However, no specific PrP signal could be detected neither for the non-chimeric nor the chimeric shortened PrP mutants, which strongly argues for a higher sensitivity of PrP W144Stop and PrP Q159Stop to digestion with PK (data not shown). To test the solubility of the PrP mutants in non-denaturing detergents, whole cell extracts of transfected N2a cells were diluted with 9 volumes of ice-cold lysis-buffer and subjected to high speed centrifugation. Supernatant proteins were methanol-precipitated, and both pellet and supernatant proteins were prepared for SDS-PAGE and Western blot analysis. Under these conditions, a higher amount of wt PrP could be detected in the supernatant fraction, indicating an overrepresentation of soluble versus insoluble wt PrP (Fig. 6B). In contrast, PrP T187K and PrP T187R showed a faintly but clearly detectable over-representation in the pellet fraction, which indicates, in comparison to wt PrP, a higher portion of insoluble protein for these PrP mutants (Fig. 6A). Interestingly, the nonsense mutants PrP W144Stop and PrP Q159Stop displayed a detergent-dependent discrimination of their truncated protein forms, which could be assayed by Western blot analysis. The more slowly migrating protein signal of PrP W144Stop and the faster migrating signal of PrP Q159Stop were represented in both the...
supernatant and the pellet fraction, with a faintly overrepresentation for both mutants in the pellet fraction. However, the lower molecular weight form of PrP W144Stop could predominantly be detected in the supernatant fraction, whereas the higher molecular weight form of PrP Q159Stop was almost exclusively represented in the pellet fraction, indicating a higher content of insoluble protein for PrP Q159Stop compared with PrP W144Stop (Fig. 6B).

Degradation Pathways of the PrP Mutants—Recent studies with proteasome inhibitors have revealed that the human PrP nonsense mutant, PrP Y145Stop, is degraded very rapidly in transfected M17 cells and that blockade of proteasomal degradation resulted in the accumulation of this PrP mutant (20). To test if application of proteasome inhibitors also affects accumulation of the other truncated PrP mutant, PrP Q159Stop, transfected N2a cells were incubated with MG-115 (30 μM), which acts as a covalent inhibitor of the trypsin- and chymotrypsin-like activities of the proteasome. A significant increase (or decrease) after application of MG-115 or lactacystin—both mutants the presented biochemical results (Fig. 7A) of the tryptophan- and chymotrypsin-like activities of the proteasome. Cell extracts were prepared after incubation of the cells with these inhibitors for 5 h, and equal amounts of total protein (100 μg each) were subjected to Western blot analysis with antisemur Can72. B, single confocal sections of N2a cells expressing chimeric GFP-PrP W144X (W144X, left panels) and GFP-PrP Q159X (Q159X, right panels) after incubation with 30 μM MG-115 or 30 μM lactacystin for 5 h. For better orientation, the nuclei are outlined. Bars, 10 μm.

To investigate the cellular phenotypes of the PrP nonsense mutants after blockade of proteasomal degradation, the expression of their chimeric PrP counterparts, GFP-PrP W144Stop and GFP-PrP Q159Stop, were assayed after incubation of cells with MG-115 (30 μM) and lactacystin (30 μM) for 5 h (Fig. 7B). Aberrant intracellular fluorescent deposits, i.e. small dot-like structures and broader signal accumulations, were clearly detectable in the cytoplasm and in the nucleus, confirming for both mutants the presented biochemical results (Fig. 7A) of an impaired degradation after incubation with proteasome inhibitors. All experiments involving the proteasome inhibitors were also done for wt PrP, PrP T187K, and PrP T187R, but no significant increase (or decrease) after application of MG-115 or lactacystin could be detected in a Western blot or in CLSM analysis (data not shown), indicating a non-proteasomal degradation of these full-length PrPs.

PrP Q159Stop Is A Short-lived Protein That Shows a Directed Influx into the Nucleus—In comparison to GFF-PrP W144Stop (and GFF-PrPAN and GFF-PrPAC, see GFF-wt Prp Is Processed and Localized Like PrPC in the “Results” section), microscopic analysis constantly revealed that GFF-PrP Q159Stop localization was almost exclusively restricted to the nucleus of transfected N2a cells. To investigate the tendency of this protein to traffic into the nuclear compartment, bleaching experiments (23) were performed to determine the fluorescent recovery after photobleaching of the nuclear signals. The fluorescent signals in the nucleus were specifically and irreversibly bleached (10 s) with high intensities of laser light (4–5 times higher than the scan mode), and the redistribution of new fluorescent signals was successively recorded under non-bleaching conditions. Very rapidly after complete bleaching of the nuclear fluorescence, new fluorescent signals appeared exclusively in the nucleus, and 8 min post-bleaching, the nuclear fluorescence was re-established similar to the prebleach situation (Fig. 8, a–d). This procedure was repeated several times, and a redistribution of new fluorescent signals was always restricted to the nucleus (data not shown), indicating a directed influx of this 41–43 kDa protein into this compartment instead of an unspecified homogeneous distribution throughout the cytoplasm and the nucleus.
The bleaching of exclusively non-nuclear regions of the cells, however, revealed a continuous decrease of the nuclear fluorescence intensity that finally, after three identical bleaching events (~4 min between each bleaching event, same bleaching conditions as above), led to the disappearance of any nuclear fluorescent signal within 10 min. Thus, loss of fluorescence in the nucleus after bleaching of large amounts of GFP-PrP Q159stop before reaching the nucleus indicates a rapid degradation of the nucleus-residing chimeras within 10 min (Fig. 8, e–h). Noteworthy, by bleaching comparable non-nuclear regions in MG-115-treated cells, the fluorescent signal in the nucleus was, in contrast to the untreated cells, still clearly detectable after three bleaching events (~4 min between each bleaching event, identical conditions as above) (Fig. 8, i–k; compare h and k) and was even easily observable after further bleaching events (data not shown), indicating a delayed degradation due to the incubation with the proteasome inhibitor. In addition, the presence of MG-115 enabled a better detection of the GFP-PrP Q159stop molecules before being located to the nucleus and revealed an arrangement of dot-like structures (Fig. 8, l), which could be hypothesized either as dislocation sites of the chimera from the ER membrane or ribosome-associated GFP-PrP Q159stop molecules (see “Discussion”).

DISCUSSION

To establish a model system for the cellular observation of prion proteins by noninvasive means, mouse PrP chimeras with the GFP marker molecule have been generated that directly permit spatial and temporal recordings of PrP localization in living N2a cells. For the construction of the chimeras, we decided to insert GFP into the N terminus of PrP, between the signal sequence and the octapeptide repeat region of PrP, since the N-terminal part of the protein has been described to be, in contrast to the structured C-terminal part, highly flexible (24). In addition, we preferred the insertion of GFP into the N instead of the C-terminal moiety to ensure the cellular phenotyping of disease-related PrP mutants that lack the complete C terminus of PrP. Recently, the production of a GFP-PrP chimera has been described that succeeded by a similar strategy of GFP insertion in the imaging of wt and mutant bovine PrPs in mammalian cell lines (25). However, due to the large amount of data existing with the well established mouse PrP model system, we decided to create mouse PrP-GFP chimeras for a more reliable comparison with the existing results.

The chimera with wt PrP (GFP-wtPrP) behaved like native PrP in so far as it was subject to the known cellular processing of PrP C. It was fully glycosylated and, following a secretory pathway, was transported via the ER/Golgi route and tethered on the cell surface by its GPI anchor. Like PrP with GFP-PrP chimera possessed no enhanced resistance to proteinase K and was therefore clearly distinguishable from PK-resistant PrP mutants and PrPSc.

Beyond biochemical or immunological analysis, which depend on the quality of the detection systems, our approach also provided the opportunity to directly examine the expression patterns of mutant PrPs in living cells and compare the cellular phenotypes with data from previous studies. In addition to the wt PrP chimera, seven chimeric PrP mutants were constructed and expressed in N2a cells. These mutants corresponded to known alterations in the prion protein associated with inherited prion diseases (26–33). Biochemical as well as cellular data have been described for four of the seven disease-related PrP mutants, i.e., the three full-length PrP mutants PrP P102L, PrP D178N, PrP E200K (7, 19) and the truncated molecule PrP Y145Stop (20) but not for PrP Q159Stop, PrP T187K, and PrP T187R (human PrPs). As described for their non-chimeric counterparts, the chimeras GFP-PrP P102L, GFP-PrP D177N, and GFP-PrP E199K (mouse PrPs) confirmed the expression pattern of glycosylated and secretory proteins, which could be detected predominantly in the Golgi apparatus and on the cell surface. Considering the biochemical characteristics of the scrapie isoform, PrPSc, we were able to show an increased resistance against proteolysis with PK, which is in agreement with published data of human brain (17, 34), transgenic mouse (35), and cell culture experiments (7) about their native PrP counterparts. Thus, even after 3 days of transient transfection and unimpeded by the insertion of GFP, the mutant PrP chimeras (but not the wild type) displayed an enhanced PK-resistance. This intriguing finding argues for a PrPSc-like conformation of the mutant PrP chimeras.

Two newly described human pathogenic PrP mutants, PrP T188K and PrP T188R, are associated with familial forms of Creutzfeldt-Jakob disease (28, 33), albeit histopathological characterizations are missing up to now. Both chimeric PrP counterparts used in this study, GFP-PrP T187K and GFP-PrP T187R, showed a cellular phenotype similar to the other disease-related full-length chimeric PrP mutants, since they were properly glycosylated and transported via the secretory pathway to the cell surface. Although a strongly reduced susceptibility of surface PrPs to PIPLC has been described in some (7, 36) but not all (19) studies for disease-related PrP mutants, we were unable to detect an impaired or delayed PIPLC reactivity of GFP-PrP T187K and GFP-PrP T187R from the cell membrane in comparison to GFP-wtPrP by direct microscopic observation. Thus, both mutants seem to be connected to the cell exterior exclusively by the GPI anchor. We cannot exclude that our findings might disregard differences between PrP membrane association in transient expression studies and long term stable cell clones. However, it seems unlikely that our results are due to the insertion of GFP, since unaffected PIPLC susceptibility has been shown for surface GFP chimeras with bovine wt and mutant PrP in stably transfected Chinese hamster ovary cell clones (25). It was suggested that the PIPLC...
resistance of several mutant PrPs is based on a physical inaccessibility of the molecules to the phospholipase, probably as a result of conformational changes that accompany conversion to the PK-resistant state (7, 36, 37). However, despite the unimpaired PIPLC susceptibility of chimeric PrP T187K and PrP T187R, both PrP mutants (native as well as chimeric) showed an enhanced PK resistance and increased detergent-insolubility compared with the wt controls, which clearly indicates a higher degree of aggregation characteristically for the disease-associated PrP isoform (7, 17, 25). Further investigations can now be conducted to determine whether chimeric mutant PrP aggregation is microscopically observable in long term stable expression systems. Recently, we described the construction of N2a cells, allowing inducible and quantitatively controllable expression of the mouse prion protein (11), which will enable us to perform more detailed studies of these and other chimeric PrP mutants.

A novel nonsense mutation of the PrP gene with a glutamine to stop codon change at amino acid 160 (Q160Stop) was found in two siblings of clinically possible or probable Alzheimer’s disease (28, 32). Western blot analysis of total protein extracts from peripheral blood leukocytes of the patients revealed an aberrant band pattern, suggesting a shortened prion protein. Our results with the mouse PrP mutant homologue PrP Q159Stop demonstrated that expression in N2a cells leads to two truncated forms of unglycosylated protein, similar to the expression pattern of the other disease-related stop mutant, human PrP Y145Stop, in M17 human neuroblastoma cells. This Gerstmann-Straussler-Scheinker syndrome-linked variant with an amber mutation at codon 145 (human PrP Y145Stop, corresponding to mouse PrP W144Stop) has been described to be not processed post-translationally and to be rapidly degraded through the proteasomal pathway (20). It has been shown for PrP Y145Stop that the higher molecular mass form represents a population of the protein that still possesses its N terminus signal peptide (20). Although we did not verify the existence of the signal peptide, a higher molecular mass form of mouse mutant PrP W144Stop and PrP Q159Stop could clearly be detected −2 kDa above the lower molecular mass forms in Western blotting, which strongly argues for a subpopulation of these mutant PrPs that conserves the signal peptide, similar to human mutant PrP Y145Stop (20). The salient histopathological features of human PrP Y145Stop are widespread PrP amyloid deposits in cerebral vessels and the presence of γ-positive neurofibrillary lesions (29). The PrP amyloid deposits have been shown to immunostain mainly with antibodies to epitopes included in the PrP sequence spanning the N-terminal 25 amino acids (38) and residues 90−147 (29), indicating that the major PrP amyloid protein is truncated at the C terminus. No histopathology has been described for the other truncated PrP mutant, human PrP Q160Stop (28). Microscopic observation of the expression in N2a cells revealed that chimeric PrP Q159Stop was located to a low extent in the cytoplasm and, with much higher signal intensity, in the nucleus. Recently, the N-terminal extremity of mature PrP has been suggested to harbor a putative nuclear localization signal (nls) reminiscent of the nls localized to cellular regions of abundant nucleic acid content, i.e. the cytoplasm and the nucleus (44). Fluorescent dot-like structures of GFP-PrP Q159Stop reminiscent of ER exit sites were clearly detectable by taking enlarged confocal images of non-nuclear regions of transfected N2a cells. It has been suggested that human PrP Y145Stop might be degraded by proteasomes on the cytosolic face of the ER, as previously reported for several secretory proteins (20, 44–46). Our model system provided first insights into the degradation pathway of the second truncated PrP mutant associated with inherited prion disease, human PrP Q160Stop. The protein seems to be sorted out from its secretory pathway at distinct sites of the ER membrane to be immediately degraded in the cytosol and nucleus.

The exceptionally strong nuclear localization of chimeric PrP Q159Stop, regardless of the inhibition or non-inhibition of proteasomal function, might argue for additional properties leading to a directed influx into the nucleus. Recently, the N-terminal region of human and ovine PrP has been described to harbor nucleic acid binding and chaperoning properties similar to those of human immunodeficiency virus-1 NCp7 and retroviral nucleocapsid proteins (47). Although the putative functional domain of PrP responsible for RNA and DNA interactions is still unknown, both truncated PrP mutants were localized to cellular regions of abundant nucleic acid content, i.e. the nucleus and ER/cytoplasm including RNA-rich ribosomes. Before this study, data on the cellular processing and localization were only available for PrP Y145Stop which had been shown to be recognized by quality control mechanisms and degraded through the proteasomal pathway (20). We were able to demonstrate that the mouse homologue of PrP Q160Stop is prevented from exit to the Golgi apparatus and transported to the cytosol and nucleus where it is also rapidly degraded in the proteasomes. Therefore, it seems reasonable to assume that expression of both truncated PrP mutants in the brain of an affected person results in similar pathological mechanisms.
that are based most probably on the impairment of proteasomal function with advanced age and/or the very slow, but continuous accumulation of truncated protein.

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REFERENCES

1. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13383
2. Hornshaw, M. P., McCormick, J. R., and Candy, J. M. (1995) Biochem. Biophys. Res. Commun. 207, 621–629
3. Brown, D. R., Qin, K., Herrs, J. W., Madlunger, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bolsen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. A. (1997) Nature 390, 684–687
4. Kramer, M. L., Kratzin, H. D., Schmidt, B., Roerke, P., Kansas, K., Schürmann, M. J., and Krause, S. (1996) J. Neurosci. 16, 734–743
5. Brown, D. R., Schulz-Schaeffer, W. J., Schmidt, B., and Kretzschmar, H. A. (1997) Exp. Neurol. 146, 104–112

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