**ALTERNATIVE TRANSLATION INITIATION GENERATES CYTOPLASMIC SHEEP PRION PROTEIN**

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Cytoplasmic localization of the prion protein (PrP) has been observed in different species and cell types. We have investigated this poorly understood phenomenon by expressing fusion proteins of sheep prion protein and green fluorescent protein (GFPPrP) in N2a cells, with variable sequence context surrounding the start codon Met1. GFPPrP expressed with the wild-type sequence was transported normally through the secretory pathway to the cell surface with acquisition of N-glycan groups, but two N-terminal fragments of GFPPrP were detected intracellularly, starting in frame from Met17. When GFPPrP was expressed with a compromised Kozak sequence (GFPPrP*), dispersed intracellular fluorescence was observed. A similar switch from pericellular to intracellular PrP localization was seen when analogous constructs of sheep PrP, without inserted GFP, were expressed, showing that this phenomenon is not caused by the GFP tag. Western blotting revealed a reduction in glycosylated forms of GFPPrP*, while the N-terminal fragments starting from Met17 were still present. Formation of these N-terminal fragments was completely abolished when Met17 was replaced by Thr, indicating that leaky ribosomal scanning occurs for normal sheep PrP and that translation from Met17 is the cause of the aberrant cytoplasmic localization observed for a fraction of the protein. In contrast, the same phenomenon was not detected upon expression of similar constructs for mouse PrP. Analysis of samples from sheep brain, allowed immunological detection of N-terminal PrP fragments, indicating that sheep PrP is subject to similar processing mechanisms in vivo.

PrP[^C] is a cell-surface glycoprotein with an essential role in the pathogenesis of transmissible neurodegenerative prion diseases (1;2). According to the prion hypothesis, a misfolded, pathogenic form of the protein (PrP[^Sc]), is the sole constituent of transmissible prions (3;4), but the molecular details and required environs for the misfolding are incompletely understood. As would be expected for a glycosyl-phosphatidyl-inositol (GPI)-anchored protein with N-linked glycans, PrP[^C] is observed at the outer leaflet of the plasma membrane, the endpoint of the secretory route. The half-time at the plasma membrane is fairly short, since the protein may undergo shedding or endocytic internalisation (5-9). Thus, PrP[^C] can be encountered throughout the secretory and endocytic routes and is also able to leave cells via exosomes derived from multivesicular endosomes (10). In agreement with this, studies of the sub-cellular distribution of PrP[^C] in mammalian brain have identified localization to the outer cell membrane, in the Golgi apparatus, and in endosomal vesicles (11;12). However, others have found that PrP[^C] is not solely associated with membranes, but, in some sub-populations of neurons, is localized to the cytoplasm (13;14). In line with the latter observations, transgenic mice expressing PrP carrying a C-terminal GFP tag demonstrated intense cytoplasmic fluorescence from a limited number (approx 1%) of the neurons in certain brain areas, such as the hippocampus (15). Immunohistochemical detection of intracellular, possibly cytoplasmic, PrP has also been reported from large mononuclear cells in the gut wall of sheep (16) and from enteric neurons in mice (17). The recent observations of pronounced cytoplasmic aggregation of PrP in pancreatic beta-cells of rats prone to development of diabetes mellitus, provide a perplexing example of non-standard PrP localization, in non-neuronal cells (18).
The flexibility observed in the subcellular localization of PrP<sub>C</sub> has been suggested to be a requirement for normal functions of the protein (14;19;20), but how cytoplasmic and nuclear variants arise has not been established. Cytoplasmic PrP could be a result of retro-translocation from the endoplasmic reticulum (ER), as part of an unfolded protein response (21-23), or from attenuated ER import of PrP under conditions of luminal stress in the ER (24;25). The finding of intact ER-targeting signal sequences on cytoplasmic PrPs (25;26), favors the latter mechanism, namely a reduced ER-import of PrP, possibly due to saturation of the ER-translocation machinery, or an overload of unfolded proteins within the ER. However, no signs of stress or pathology could be detected in neurons of wild-type mice expressing cytoplasmic PrP (14), which led to the suggestion that the cytoplasmic appearance of PrP could constitute a physiologically relevant, but minor, pathway for the protein.

Forced cytoplasmic expression of PrP in transgenic mice (22) and in the nematode Caenorhabditis elegans (27) resulted in neurodegenerative disease, suggesting that toxic mislocalization of PrP could be part of the pathogenetic mechanism in prion diseases (28). However, transgenic mice expressing cytoplasmic PrP, on a PrP-null background, developed cerebellar atrophy, but were resistant to experimental prion infection (29), suggesting that cytoplasmic PrP is unlikely to serve as substrate for prion replication. Furthermore, data obtained from transgenic mice expressing an anchorless secretory PrP show that, although these mice accumulate PrP-containing amyloid plaques upon challenge with PrP<sub>Sc</sub>, they fail to develop clinical prion disease (30). Thus, membrane-attached PrP appears to be a prerequisite for development of prion-derived neurodegeneration.

In eukaryotes, ribosomes bind specifically to linear mRNAs carrying a 7-methylguanosine 5'-end cap and slide along the mRNA in the 5' – 3' direction until they encounter the first start codon (AUG), from which the protein translation starts exclusively. Therefore, eukaryotic mRNAs are generally monocistronic. However, deviations from this standard principle have been reported, in which protein translation is initiated at alternative start codons either up or downstream from the primary AUG. The best characterized mechanism is known as context-dependent leaky ribosomal scanning (LRS) (31). This cap-dependent mechanism is particularly operative when the optimal (5'-GCCRCCaugG-3') sequence context surrounding the first AUG codon is compromised, most notably at positions R<sub>3</sub> (R = purine, A or G, but optimally G) and G<sub>4</sub> (32;33).

In this work, we report that in a cell culture system, sheep PrP mRNA displays a tendency to allow alternative translation initiation through LRS. Met<sub>17</sub> serves as an internal in-frame alternative start codon giving rise to PrP with a severely shortened ER-targeting peptide.

While the LRS mechanism is active in sheep PrP, it appears to occur much less in mouse PrP (34). The molecular explanation and possible patho-physiological relevance of these observations in relation to PrP function await further studies. Interestingly, during the review process of this paper, observations of cytoplasmic PrP similar to some of those described herein, were reported for human and hamster PrP (35).

MATERIALS AND METHODS

Construction of plasmids-The construction of plasmids coding for PrP* and GFP<sup>PrP</sup>*, has been described previously (36). The PrP and the GFP<sup>PrP</sup>, which contain the naturally occurring sheep Kozak sequence (5'-GTC ATC preceding the start codon ATG, otherwise similar to PrP and GFP<sup>PrP</sup>*), were generated using the PrP* and the GFP<sup>PrP</sup>* constructs as templates, standard cloning techniques, and the following primers: forward (5'-TAGCCTCGAGGTCATCATGGTGAAAAGCCACATAGG-3') and reverse (5'-CGACTCTAGAGTACTATGAGAAAAATGAGG-3'). The GFP<sup>PrP<sub>MIR</sub></sup> and GFP<sup>PrPV2A</sup> constructs were made using the same 3'-primer, but the forward primers were: (5'-TAGCCTCGAGGTCATCATGGTGAAAAGCCACATAGG-3') and (5'-TAGCCTCGAGGTCATCATGGTGAAAAGCCACATAGG-3'), respectively. Insertion of the PCR product into the multiple cloning site of the pcDNA 3.0 vector (Invitrogen, CA, USA), was done using standard cloning...
techniques and the restriction enzymes XhoI and XbaI. The GFPPrP<sub>M17T</sub> was made by site-directed mutagenesis with the GFPPrP as template and the following primers: forward (5’-GGTCTCTTTTGCGCCACATTGAGTGACGTG-3’) and reverse (5’-CCACGTCACTCCAGTGGCCACAAAAGAAGACC-3’). GFPPrP<sub>Δ2-24</sub> was made by the forward (5’-CTGGAATTCAGTATGAAGAAGCGACCAAAACCTGGC-3’) and reverse (5’-ATTAGCTCGAGTACTATCTACTATGAAAAAT-3’) primers, and ligated into the pEGFP-C1 with the restriction enzymes NheI and XhoI. GFPmoPrP, was kindly provided by Dr. Otto Windl (Veterinary Laboratory Agency, Weybridge, UK), and its cloning has previously been described (37). GFPmoPrP* was generated from the GFPmoPrP with the following primers: forward (5’-ATATCTCGAGTTCTATGGCGAACCTTGG-3’) and reverse (5’-TTAATCTAGAATCTAGTGGATCCCCCG-3’). The restriction enzymes XhoI and XbaI were used to insert the amplified products into multiple cloning sites in the pcDNA 3.0 vector. All mutations were verified by sequencing.

Cell culture and DNA transfection-The mouse neuroblastoma cell line N2a was kindly provided by Dr. Jörg Tatzelt, (Ludwig-Maximilians-University Munich, Germany). N2a cells were cultured at 37°C under 5% CO₂ in minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) complemented with 10% fetal calf serum (Euroclone, Siziano, Italy), 1% penicillin/streptomycin, non-essential amino acids, and pyruvate (Cambrex, Charles City, IA, USA). Transfection of N2a cells was performed in Opti-MEM (Invitrogen) with Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer’s instructions. Transiently transfected cells were harvested 24 h after transfection. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% (w/v) Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA), supplemented with proteinase inhibitor tablets (Roche, Complete, Hoffman-La Roche AG, Basel, Schweiz) for 1 h on ice. Treatment of cells with the proteasome inhibitor lactacystin (LC) at 2.5 µM concentration was performed in Opti-MEM for 18 h.

Antibodies-Antibodies used: P4 (Food Diagnostics, Oslo, Norway), anti-GFP 6556 (Abcam, Cambridge, UK). The polyclonal anti signal-peptide antibody (Anti-SP) which binds to the ER-targeting signal of PrP (38) was a generous gift from Dr. David A. Harris (Washington University School of Medicine, MO, USA). The polyclonal antibody R505, which binds to 102WNKPS106 in the sheep PrP (39;40) was a kind gift from Dr. Jan Langeveld (Leystad, The Netherlands). In Western blots, primary antibodies were used at 0.05 – 0.2 µg/ml concentrations. Secondary antibodies, goat-anti-mouse, conjugated with horseradish peroxidase (HRP, BioRad, Hercules, CA, USA and Invitrogen) or alkaline phosphatase (ALP, Invitrogen) and goat-anti-rabbit ALP (Invitrogen) were used at 0.05-0.3 µg/ml concentrations.

Enzymatic deglycosylation and Western blotting-Cell lysates were centrifuged for 5 min at 13000 rpm to remove aggregates and nuclear components. For enzymatic deglycosylation, samples were treated with N-glycosidase F (PNGase F, New England Biolabs) for 1 h at 37°C, with buffers recommended by the manufacturer. The protein concentration in cell lysates was measured with a dye-binding assay (BioRad), to ensure similar loading between lanes. Samples were boiled in SDS sample buffer (NuPAGE, Invitrogen) under reducing conditions and separated by electrophoresis on precast 12% Bis-Tris polyacrylamide gels, with XT-MOPS as running buffer (BioRad). The proteins were electro-blotted onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences, Amersham, UK) by a semi-dry blotter (Trans Blot Semi-Dry, BioRad). Membranes were blocked by incubation with 5% (w/v) fat-free dried milk (BioRad) in TBS for 1 h at room temperature (RT). Incubations with antibodies were performed in TBS with 0.1% Tween-20 and 1% (w/v) fat-free dried milk overnight at 4 °C for primary antibodies and for 1 h at RT for secondary antibodies. Bands were visualized by enhanced chemiluminescence (ECL Plus, Amersham Biosciences), captured by ECL Hyperfilm (Amersham Biosciences), or by fluorescence scanning with a variable mode imager (Typhoon 9200, Amersham...
Biosciences) when secondary antibodies labelled with ALP were used.

**Immunoprecipitation for MALDI-MS/MS analysis-** Post-nuclear supernatants from transiently transfected cells were immunoprecipitated with the monoclonal antibody P4, covalently linked to Dynabeads M-270 Epoxy (Invitrogen). Beads were diluted in 2 ml dimethyl formamide to a final concentration of 2 x 10⁶ beads/ml. The paramagnetic beads were covalently coated with P4 as follows: beads were washed and resuspended in 55 µl 100 mM sodium phosphate buffer, pH 7.4, complemented with 15 µg P4 and 0.1% bovine serum albumin (BSA). After thorough mixing by vortexing, 35 µl 3 M ammonium sulphate was added. After incubation for 48 h at RT with gentle mixing, the beads were washed four times in PBS with 0.1% BSA. A sub-sample of 1 ml cell lysate was added to a pellet of coated beads, corresponding to 27 µl bead suspension, and incubated at RT for 30 min before washing three times in PBS. The resulting bead pellet was boiled for 5 min in 20 µl of SDS sample buffer. The beads were removed by use of a magnet and the supernatants electrophoresed as previously described. Coomassie-stained bands of interest were excised by scalpel for in-gel digestion in 20 µl of 50 mM ammonium bicarbonate, pH 7.8 with either 0.1 µg of trypsin (Promega, Madison, WI, USA) or 0.2 µg chymotrypsin (Sigma-Aldrich Norway A/S, Oslo, Norway). The enzymatic digests were purified using ZipTip µ-C18 (Millipore, Billerica, MA, USA) and the eluates dried using a Speed Vac concentrator (Savant, Holbrook, NY, USA). The peptides were dissolved in 0.5 µl 0.2% trifluoroacetic acid/acetonitrile (2:1) and mixed with 0.5 µl 20 mg/ml α-cyano-4-hydroxycinnamic acid in 0.2 % aqueous trifluoroacetic acid/acetonitrile (2:1). The samples were applied to a stainless steel sample holder and, after drying, introduced into the ULTRAFLEX II (Bruker Daltonics, Bremen, Germany) MALDI-TOF/TOF mass spectrometer and analyzed in the MS mode (for generation of peptide mass fingerprints) as well as in the TOF/TOF mode (for fragmentation analysis). MS spectra were transformed into peak lists using the software FlexAnalysis version 2.4. (Daltonics, Bremen, Germany). The software program PeptideMass (http://au.expasy.org/tools/peptide-mass.html) was applied to predict tryptic and chymotryptic peptides. Matching peptide masses within the peptide mass fingerprints were selected for MALDI-MS/MS analysis. Fragments b and y-ions were calculated using the software program Fragment Ion Calculator (http://db.systemsbioinformatics.net:8080/proteomics/FragmentIonServlet.html).

**Immunoprecipitation for Western blot analysis-** Post nuclear supernatants of transiently transfected N2a cells were rolled with 2 µg of monoclonal antibodies P4 or Saf-32, overnight at 6°C. A Protein G agarose (Kirkegaard & Perry Laboratories, Maryland, USA) slurry (50/50) was prepared in 50 mM Tris, 150 mM NaCl, 0.05% Triton X-100, pH 7.5 and 1% BSA as described by the manufacturer and incubated on a roller overnight at 6°C. Aliquots of 20 µl Protein G slurry were added to the samples and mixed on a roller for 3 h at 6°C. Thoroughly washed agarose pellets were boiled in SDS sample buffer for 5 min before centrifugation at 13 000 rpm for 2 min at RT. Supernatants were subjected to SDS-PAGE and Western blotting. Western immune blots were developed with mouse TrueBlot ULTRA, HRP anti-mouse-IgG (Bioscience) after incubation with monoclonal primary antibodies, while the polyclonals anti-SP and R505 were developed with anti-rabbit IgG conjugated to alkaline phosphatase (BioRad).

**Microscopy-** Cells transiently transfected with un-tagged PrP were grown on glass coverslips (Assistent) in a 24 well plate (Sarstedt). After 24 h, cells were fixed in PBS supplemented with Ca²⁺ (0.02 mM) and Mg²⁺ (0.2 mM) (PBS*) and 3.7% formaldehyde before permeabilization with 0.1% Triton X-100, blocking in 5% fat-free dried milk in PBS and incubation for 1 h with primary antibody P4 in PBS with 1% fat-free dried milk. After washing with PBS, cells were incubated with goat-anti-mouse secondary antibody labelled with alexa-488 fluorochrome (Molecular Probes) before confocal microscopy. Cells transiently transfected with various GFPPrP constructs were grown on glass slides (LabTek II Chambered coverglass, Nunc), and analyzed after 24 h by the fluorescence protease protection assay (41). Briefly, cells were washed with KH buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM MgCl₂) at 37°C and treated with either digitonin (40 µM) to permeabilize the plasma membrane, or trypsin,
(4 mM) or both, in the presence or absence of 1% (vol/vol) Triton X-100. Non-saturated images were captured by a Plan-Apochromat 63/1.4 oil objective in a Zeiss laser scanning confocal microscope (Zeiss Axiovert 200M fluorescent inverted microscope, equipped with a LSM 510 laser confocal unit and 488 nm argon laser and 546 nm helium/neon laser, Carl Zeiss, Jena, Germany).

Crude preparations from sheep brain-
Purification of synaptosomal/microsomal membrane fractions from sheep brain was performed as described by (42). Briefly, immediately after euthanasia, the brain was removed and 40 g was homogenized in a Dounce all-glass homogenizer in 200 ml of ice-cold bicarbonate-buffer containing 0.32 M sucrose, 0.5 mM KCl, 1 mM MgCl₂, 1 mM NaHCO₃ and protease inhibitor tablets (Complete, Hoffman-La Roche AG, Basel, Schweiz). After initial low-speed centrifugations and rehomogenization, supernatants were combined and centrifuged at 100,000 g on a 0.85 M sucrose cushion at 4°C for 1 h. The resulting pellet was highly enriched in PrP and used for Western blot analysis.

RESULTS

Sheep PrP fused with green fluorescent protein (GFP)- We have generated a large number of sheep GFPPrP gene constructs, in which GFP is positioned between amino acid 43 and 44 in the N-terminal region of PrP (Fig. 1). Our initial observations indicated remarkable differences in intracellular trafficking of both un-tagged PrP and GFPPrP fusion variants. These enticed us to conduct a more systematic study, particularly of the region surrounding the start codon triplet (AUG), including the Kozak sequence. Constructs (PrP* and GFPPrP*) with compromised Kozak sequence were expressed in N2a cells, giving diffuse intracellular staining, indicating cytoplasmic localization of these PrP variants (Figs. 2A, 3A). For comparison, wild-type PrP and GFPPrP (intact Kozak sequence) displayed a typical cell surface localization, with some of the protein localized to intracellular vesicular compartments. Western blot analysis revealed that mono- and di-glycosylated forms of PrP prevailed when PrP with intact Kozak sequence was expressed, while for PrP*, un-glycosylated and mono-glycosylated forms dominated. An N-terminal fragment of about 8 kDa was faintly visible in untreated cells, and more clearly visible after treatment with the proteasome inhibitor LC (Fig. 2B). Next, we expressed a construct lacking the region coding the ER-targeting signal peptide (GFPPrP₃₂₋₂₄) to compare the subcellular localization of the corresponding protein with that of GFPPrP*. The two proteins displayed similar distribution patterns in N2a cells (Fig. 3A).

The differential localization of GFPPrP fusion protein variants was verified by the fluorescence protease protection assay. Cells expressing GFPPrP with a typical plasma membrane appearance lost the corresponding fluorescent signal after 30 sec of trypsin treatment (Fig. 3B), indicating that the protein was attached to the outer leaflet of the plasma membrane. GFPPrP*, however, was trypsin resistant, even after 2 min of treatment, but the fluorescence was reduced after digitonin-mediated permeabilization of the plasma membrane, and was rapidly abolished upon combined treatment with trypsin, digitonin and Triton X-100 (Fig. 3B). This indicates that GFPPrP* was localized intracellularly, largely in a freely diffusible form, and unprotected by endomembranes that are resistant to digitonin treatment. Furthermore, proteinase resistant aggregates were not observed.

We also investigated biochemically the intracellular fate of newly synthesized GFPPrP variants. PrP normally enters the lumen of the ER co-translationally, to obtain one or two N-glycan groups. Such variants are clearly the predominant products for GFPPrP in N2a cells (Fig. 4A and B, lane 1). The variant lacking the entire ER signal peptide (lane 3) was expressed as a full-length, non-glycosylated variant, but also appeared as a truncated doublet derived from the N-terminal region. Similarly, this doublet was the dominant feature for GFPPrP* (lane 2), which also displayed a full-length non-glycosylated variant, and only faint bands for mono- and di-glycosylated GFPPrP. The doublet was recognized by the P4 antibody, but not by antibodies recognizing epitopes situated closer to the C-terminus of PrP (36). Epitope mapping (Figs. 4 and 6) and MALDI-MS/MS analysis (Fig. S1, Supplementary Material) enabled us to narrow the C-termini of the truncation doublet to residues 104 and 115, with an error margin of 2-3 residues. Since, these proteolytic products were prominent in
the variant lacking the signal sequence, and also clearly observable for the wild-type construct (Fig. 4A), we could not deduce whether their appearance for GFPPrP* resulted from compromised ER import or from successful ER import, followed by retrograde transport to the cytoplasm. We therefore investigated whether the proteolytic fragments could be detected by an anti-SP antibody. The doublet bands obviously contained the SP, indicating that GFPPrP* did not encounter the ER signal peptidase prior to proteolytic processing in the cytoplasm (Fig. 4B). Treatment of cells with the proteasome inhibitor LC, led to a slight increase in full length un-glycosylated forms (Fig. 2B and 4B, arrowheads) and a weak decrease in staining of the N-terminal doublet, indicating that proteasomal degradation of full length GFPPrP could occur.

Alternative translation initiation at Met17 generates cytoplasmic PrP- To test the possibility that Met17 could operate as an internal, in-frame translation initiation site in sheep PrP, we made two mutants of the GFPPrP construct in which we substituted Met1 (GFPPrPM1R) and Met17 (GFPPrPM17T), respectively. Transient expression of the GFPPrPM1R construct mimicked our observations in striking detail after expression of the GFPPrP* plasmid, not only with the dominant intracellular fluorescence signal (Fig. 5A, left panel), but even more so in Western blot analysis, in which the characteristic truncation doublet with intact anti-SP reactivity could be detected (Fig. 5B, lane 2, all panels). This observation allowed us to conclude that the truncation doublet starts at Met17 which preserves the anti-SP epitope except for the N-terminal residue Thr16 (T). Thus, removal of Met1 elicited production of cytoplasmic GFPPrP, starting at Met17. However, the definitive experiment would be to test whether removal of Met17 could block this minor pathway for sheep PrP. Indeed, expression of the GFPPrPM17T plasmid gave rise to a membranous, pericellular fluorescence signal (Fig. 5A), but, importantly, it resulted in complete removal of the truncation doublet (Fig. 5B, lane 3, upper and bottom panels). Even over-exposure (bottom panel) failed to detect the truncation doublet, which, however, was present after expression of the GFPPrP plasmid with the wild-type Kozak sequence surrounding Met1 (lane 4, upper and lower panels). This illustrates that a minor cytoplasmic pathway was operative when this construct was expressed. Expression of the GFPPrPV2A plasmid, in which the second amino acid residue Val (V) in sheep is substituted with Ala (A), as in primate and rodent PrPs, failed to produce any significant difference from the wild type (GFPPrP) or GFPPrPM17T by fluorescence microscopy (Fig. 5A), or from GFPPrP in Western blots (Fig. 5B, lane 4, upper and lower panels). This was investigated because this amino acid substitution theoretically corresponds to an improvement in the sequence surrounding Met1, as the +5T is substituted with the slightly more ideal +5C. However, the assays used here failed to detect any consistent effect of this change. Notably, detection of immunoprecipitated samples with the anti-SP antibody, displayed a lower sensitivity as compared with the P4 antibody, which appears extremely efficient in Western blotting. This difference in sensitivity is evident when comparing lanes 4 and 5 in Fig. 5B. Thus, direct detection of the truncation doublet by P4 in cell lysates (Fig. 5B, upper panel) proved to be a reasonably sensitive and very simple assay for the detection of cytoplasmic GFPPrP, driven by constructs described herein.

Failure to provoke alternative translation start in mouse PrP- The alternative translation initiation we observed for sheep PrP led us to investigate whether the same phenomenon occurs for PrP from another species. We therefore used mouse GFPPrP constructs, which are only a few amino acids different from the sheep constructs at the insertion-site for GFP (Fig. 6A, details in methods). As shown in Fig. 6A, the ER-targeting SP in mouse PrP is 2 residues shorter than the sheep equivalent, with additional coding differences at 8 positions out of 15. For mouse PrP, Met15 corresponds to Met17 in sheep PrP and could theoretically serve as an internal translational initiator. Two constructs were made, one with a wild-type Kozak sequence surrounding Met1 (GFPmoPrP) and one (GFPmoPrP*) with a severely compromised Kozak sequence (Fig. 6A). Expression of these constructs failed to reveal any differences in cellular distribution of the fluorescence signal (Fig. 6B) and in Western blots (Fig. 6C). Since the P4 antibody does not detect mouse PrP, it was substituted by an anti-GFP antibody in this assay. This antibody also detects the N-terminal truncation doublet in sheep PrP with
adequate sensitivity. However, no traces of the truncation doublet could be found after expression of the mouse constructs, indicating that if a cytoplasmic variant of GFPPrP was expressed, its level was below the sensitivity of our assay. The somewhat weaker Western blot signal for GPFmoPrP*, as compared with GPFmPrP (Fig. 6C), could indicate that the compromised Kozak sequence resulted in a general reduction of translation efficiency. Interestingly, Western blot analysis of crude sheep brain homogenates allows detection of N-terminal fragments that correspond well with those observed in cell culture (Fig. 7, lane 6). However, discriminating these from fragments generated by other PrP processing events remains challenging.

In summary, we have demonstrated that in N2a cells, sheep PrP mRNA with a proper Kozak sequence around the first start codon allows alternative initiation of translation through LRS. The alternative start codon results in PrP with a severely shortened ER SP that remains in the cytoplasm and consequently is subject to proteolytic breakdown. This mechanism, detected for sheep PrP, occurs at a much lower level for mouse PrP, when this protein is expressed in the same cell culture system.

**DISCUSSION**

In this work, we investigated whether mechanisms related to protein translation initiation could contribute to the generation of cytoplasmic PrPs. In order to address this question, we generated sheep PrP constructs, in particular GFPPrP chimeras in which the nucleotide sequence surrounding the first initiator codon (Met1) was compromised. This nucleotide sequence, known as the Kozak sequence, has been comprehensively studied for its influence on protein translation (31). A peculiarity of the constructs that lack an intact Kozak sequence, was that they resulted in a shift in cellular localization of PrP with a severely shortened ER SP that remains in the cytoplasm and consequently is subject to proteolytic breakdown. This mechanism, detected for sheep PrP, occurs at a much lower level for mouse PrP, when this protein is expressed in the same cell culture system.

Terminal of Cys\textsubscript{34} in sheep PrP and destroys the anti-SP epitope. PrP starting at Met\textsubscript{17}, the alternative down-stream translation start, still harbors the epitope for the anti-SP antibody. Cultivation of transfected cells in the presence of the proteasome inhibitor LC resulted in a slight increase of cytoplasmic full length PrP with a corresponding reduction of the truncation doublet (Fig. 4B), in agreement with previous observations of proteasomal degradation of cytoplasmic PrP species (26).

The N-terminal PrP doublet was also clearly detectable when the Kozak sequence was intact (GFPPrP). This finding triggered us to consider whether the truncation doublet signified a minor, constitutive pathway in sheep PrP, generating cytoplasmic PrP. Of note, expression of GFPPrP revealed no indication of cytoplasmic PrP as judged by fluorescence microscopy, which reflects the relatively low sensitivity of this assay in searching for minor protein pathways. As pointed out by Levine and co-workers (34) a minor protein pathway must reach 10-20% of the total amount, before direct detection of mislocalized GFP-tagged targets by fluorescence is possible. Fluorescence protease protection was used to confirm the gross cellular localization of the two variants, GFPPrP and GFPPrP*, with an extracellular signal from GFPPrP (Fig. 3 A and B) and an intracellular, slowly diffusible trypsin-sensitive form for GFPPrP*. Taken together, these data prompted the question whether the internal methionine found at residue 17 (Met\textsubscript{17}) in ruminant PrPs could function as an alternative in-frame initiation site for translation. In support of this possibility, mutational substitution of Met\textsubscript{17}, maintained the membranous PrP signal and completely abolished the N-terminal doublet, while substitution of Met\textsubscript{1}, in the presence of Met\textsubscript{17} produced a picture indistinguishable from that seen with the GFPPrP*, with dispersed intracellular PrP-signal and a dominant N-terminal truncation doublet in Western blots. These observations, in particular the effects seen by sequence manipulations surrounding Met\textsubscript{1}, are exactly those which would be expected with the LRS mechanism.

Context-dependent LRS is a well characterized mechanism (23). A cap-independent mechanism based upon internal ribosomal entry sites (IRES), originally described for viral mRNAs with long and complex 5’ non-translated regions, has also
been proposed to play a role in cellular mRNAs (44), but the evidence has been questioned (45). An analysis of more than 22 200 human mRNAs showed that only 37.4% of the genes adhered to the optimal AUG sequence context and that 12.5% of the genes lacked both R_{3} and G_{+4} which could potentially lead to strong LRS (46). Low level LRS is difficult to detect and is probably a vastly underestimated phenomenon.

The compromised sequence surrounding Met_1 in the G^{PrP}_{*} plasmid apparently provoked strong LRS and correspondingly led to a dominating shift in PrP localization as compared with the same construct, but with wild-type Kozak sequence, G^{PrP}, in which the expected membranous localization of PrP was seen. In ruminant and mink PrPs the second amino acid is V, while in primates and rodents it is A. To investigate whether this difference could be part of the LRS mechanism, a G^{PrP}_{V2A} construct was made. However, this substitution did not influence the occurrence of LRS as judged by detection of the N-terminal doublet (Fig. 5A and B).

The observations with sheep PrP inspired further search for LRS in PrP mRNA from another species. Plasmids encoding mouse PrP carrying GFP in the N-terminal tail, almost identical to the sheep GFP-tagged PrPs described here, were acquired, one with compromised (G^{PrP}_{moPrP*}), and one with wild-type (G^{PrP}_{moPrP}), Kozak sequences (Fig. 6A). However, no difference could be observed between the two constructs, neither by fluorescence microscopy nor in Western immune blots (Fig. 6 B and C). This was in contrast to the shift in cellular localization seen for the sheep PrP, indicative of substantial species variation in this phenomenon. In mouse PrP, Met_{15} could theoretically serve as an internal in-frame translational initiation site analogous to Met_{17} in sheep PrP. Our data, however, suggest that LRS appears less frequently in mouse PrP. The mechanism behind this species difference is not known, but could be related to structural nuances between the mRNAs that could influence the fidelity of start codon selection. Regardless, our data with G^{PrP}_{moPrP} agree with those from previous analysis of LRS in mouse PrP in which a very sensitive luciferase-based assay revealed that LRS occurs at a low level in mouse PrP. However the assay used herein is probably insufficiently sensitive to detect this level (34). The species differences observed could indicate that down-stream structural elements in mouse PrP retard the 40S ribosome to ensure that translational initiation is adequate, including when the initiator context is sub-optimal.

The observations of cytoplasmic PrP only in certain sub-sets of neurons (14;15) suggest that the cellular localization of PrP can vary between cell types, influenced by cell-specific factors that remain to be identified. Concerning the cell-specificity of the alternative translation initiation observed here for sheep PrP, similar data have been obtained with different cultured cell-types, such as canine kidney cells (MDCK), human neuroblastoma (SH-SY5Y), and colon cancer (LoVo) cells (36;47), (data not shown). Thus, alternative translation initiation seems to operate constitutively in sheep PrP. However, our data cannot exclude that this process is subject to regulation. Several in vitro studies have shown that under normal conditions, a small portion of newly synthesized PrP, with apparently intact ER-SP, localize to the cytoplasm and is subject to proteasomal degradation (26;28). It was speculated that this seemingly abnormal, but dynamically fluctuating, metabolism of PrP could contribute to physiological processes, related to blood glucose regulation, and not merely a pathological aberration (18).

The full in vivo relevance of LRS-driven cytoplasmic generation of sheep PrP reported here remains to be established. One way of analyzing this further would be to generate transgenic mice carrying sheep PrP, with and without strong LRS, and compare the in vivo cellular handling of PrP in these, as well as the response to prion infectivity, upon experimental challenge. However, it is noteworthy that in a parallel and independent study to this report, Juanes and co-workers (35) found a nucleo-cytoplasmic localization of human and hamster PrP generated by internal translational start at Met_8 and Met_{15} respectively. Moreover, they found that expression of a mutated hamster PrP with augmented translational start at Met_{15} in CHO cells appeared to suppress cellular growth. We did not observe this phenomenon with any of
the constructs reported here, regardless the level of cytoplasmic PrP, whether or not GFP had been inserted.

Inappropriate expression of cytoplasmic PrP in transgenic mice (22) or C. elegans (27) revealed severe neuropathological effects of the mislocalized PrPs, but results from cell culture studies of cytoplasmic PrP have been contradictory, with reports of high toxicity (22), moderate toxicity (48), absence of toxicity (49), or even neuroprotective effects (20). In order to evaluate the possible toxic interference of cytoplasmic PrP with the biogenesis of normal PrP, Norstrom and co-workers (29) crossed the original transgenic line expressing cytoplasmic PrP, TG1D4 (Prnp+/+) (22) with a PrP-knockout (Prnp0/0) line to generate a TG1D4 (Prnp0/0) line, expressing cytoplasmic PrP on a PrP-null background. No differences were observed between the two lines, and mice from both lines developed spontaneous cerebellar atrophy without generation of prion infectivity, spongiosis or PrPSc. Moreover, the TG1D4 (Prnp0/0) line of mice appeared resistant to prion inoculations, suggesting that cytoplasmic PrP could not serve as substrate for prion replication, possibly due to the physical separation of the cytoplasm from the extracellular environment (29). Thus, although cytoplasmic PrPs have been shown to harbor pathogenic potential in transgenic models, possibly mediated through membrane perturbations (50) a significant role in the prion pathogenesis or replication seems unlikely.

LRS-driven production of a cytoplasmic sheep PrP surely constitutes a burden on the cell’s capacity to dispose of mislocalized protein, which, even if occurring at very low levels, could create significant problems. A slight drop in the cell’s clearance of mislocalized protein could potentially lead to cytoplasmic build-up of PrP or PrP-fragments, with the inherent risk of misfolding, aggregation and toxicity. However, in the cell culture models used here, no decrease in cellular viability or growth-rate could be observed, even under conditions of strong LRS. The possibility that LRS-generated cytoplasmic isoforms of PrP could be part of as yet undiscovered functional pathways for PrP, should not be excluded until properly explored.

In conclusion, we have discovered that sheep PrP mRNA displays a tendency to allow alternative translation initiation through LRS, in cell culture systems. This also operates when wild-type Kozak sequences surround the first start codon. Met17 serves as the internal in-frame alternative start codon, resulting in PrP with severely shortened ER-targeting peptide, which translocates poorly into the ER. This mechanism is probably also active in hamster and human PrP (35), while it appears to occur at a much lower level in mouse PrP (34, and this report). Taken together, our data provide new insights into the intriguing observations of minor non-membranous cellular pathways for PrP.

Reference List


FOOTNOTES
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The abbreviations used are: Ab, antibody; Ala, alanine; ALP, alkaline phosphatase; BSA, bovine serum albumin; CCC, central charge cluster; ER, endoplasmic reticulum; GFP, green fluorescent protein; GPI, Glycosyl-phosphatidyl-inositol; HRP, horseradish peroxidase; IRES, internal ribosomal entry sites; LC, lactacystin; LRS, leaky ribosomal scanning; Met, Methionine; moPrP, mouse prion protein; NCC, amino-terminal charge cluster; PrP, prion protein; GFPPrP, prion protein tagged with green flourescent protein; PrP<sup>C</sup>, normal cellular isoform of the prion protein; PrP<sup>Sc</sup>, abnormal partly protease resistant isoform of the prion protein; RT, room temperature; SP, signal peptide; Val, valine;

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FIGURE LEGENDS

**FIGURE 1. Sheep GFP<sub>GFPPrP</sub> constructs**
In the sheep PrP primary structure, an N-terminal ER-targeting signal peptide (SP, residues 1-24) precedes a stretch of positively-charged residues (NCC, residues 25-30). The central domain of the protein consists of another charge cluster (CCC, residues 104-115), and a hydrophobic core (HC, residues 115-134). The globular C-terminal domain (135-233) contains two possible N-glycosylation sites (black lollipops, residues 184, 200) and a C-terminal GPI-anchor addition sequence (residues
Epitopes for the anti SP (PrP residues 16-29), P4 (PrP residues 93-99) and R505 (PrP residues 102-106) are indicated. Green fluorescent protein (GFP) was inserted between amino acid 43 and 44 in all sheep constructs. The start codons Met1 and Met17 are highlighted in bold and underlined. The nucleotide sequence context surrounding the start codon (ATG) is given below the amino acid sequence.

**FIGURE 2. Sheep PrP expressed in mouse neuroblastoma cells.**

Un-tagged sheep PrP and PrP* (corresponding to GFPPrP and GFPPrP* but lacking the inserted GFP) were transiently transfected into mouse neuroblastoma (N2a) cells. Treatment with the proteasome inhibitor LC was performed for 18 h. A: Immunofluorescence using P4 and Alexa-488 conjugated antibody, the images were captured by a laser confocal microscope at 63 x magnification. B: Sheep PrP and PrP* were transiently expressed in N2a cells, then PrP and PrP* from both untreated and LC (2.5 µM) treated cells were immunoprecipitated by the P4 Ab, followed by Western blot analysis using the same Ab.

**FIGURE 3. Sheep GFPPrP constructs expressed in mouse neuroblastoma cells.**

A: Mouse neuroblastoma (N2a) cells were transiently transfected to express GFPPrP, GFPPrP* or GFPPrP_{52-24}, and the images were captured at 63 x magnification using a laser confocal microscope. B: N2a cells transiently transfected to express GFPPrP or GFPPrP* were treated as indicated with trypsin for 30 s or 2 min, respectively. N2a cells expressing GFPPrP* were then treated with digitonin for 1 or 2 min, or treated with digitonin, trypsin and Triton X-100 for 1 min. The scale bar is 10 µm.

**FIGURE 4. Sheep GFPPrP variants in mouse neuroblastoma cells.**

A: The sheep GFPPrP constructs coding GFPPrP, GFPPrP* or GFPPrP_{52-24} were transiently expressed in N2a cells. Cell lysates were analysed by Western blot using P4 (upper panel), or GFPPrP variants were immunoprecipitated with P4 prior to Western blot analysis using R505 (middle panel) or Anti-SP (lower panel). B: Cells cultivated overnight in the absence or presence of 2.5 µM LC were analyzed by Western blot using P4. Arrowheads indicate the unglycosylated full length GFPPrP variants.

**FIGURE 5. Mutated translation initiation sites in sheep GFPPrP.**

A: Mutations around the translation initiation site in GFPPrP were introduced, and the constructs coding GFPPrP_{M1R}, GFPPrP_{M17T}, or GFPPrP_{V2A} were transiently transfected into N2a cells. B: Cell lysates from N2a cells transiently expressing GFPPrP, GFPPrP*, GFPPrP_{M1R}, GFPPrP_{M17T}, or GFPPrP_{V2A} were analyzed by Western blot using P4 (upper panel), or GFPPrP was immunoprecipitated with P4 followed by Western blot with anti-SP (middle panel) or P4 (lower panel).

**FIGURE 6. Mouse GFPPrP constructs expressed in mouse neuroblastoma cells.**

A: Schematic alignment of sheep and mouse GFPPrP coding constructs with single letter ER-targeting sequences, and identical amino acids indicated by dotted lines between the constructs. B: N2a cells were transiently transfected to express GFPmoPrP and GFPmoPrP*, and compared by confocal microscopy. C: Cell lysates from transiently transfected N2a cells with GFPmoPrP and GFPmoPrP* were analyzed by Western blot, using anti-GFP. The scale bar is 10 µm.

**FIGURE 7. PrP from crude sheep brain homogenate.** Normal sheep brain homogenates were either treated with PNGase F or untreated, and analyzed by Western blot using P4.
Figure 1

PrP

GFPPrP

gtc atc ATG gtg

GFPPrP*

aat tct ATG gtg

GFPPrP_{Δ2-24}

gtc atc ATG gtg

GFPPrP_{M1R}

gtc atc aga tct

GFPPrP_{M17T}

gtc atc ATG gtg

GFPPrP_{V2A}

gtc atc ATG gcg
Figure 2B

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Full length PrP

N-terminal fragment
Figure 3B

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Figure 4A

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**Full-length GFPPrP**

**N-terminal doublet**

Anti-SP

R505

P4

Ab

kDa

-60

-50

-40

-50

-40

-40
Figure 4B

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Ab P4

Full length GFPPrP

N-terminal doublet
Figure 5B

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Full length GFPPrP

N-terminal doublet

IP mAb P4

Anti-SP

P4
Figure 6A

\[ \text{GFP}_{\text{PrP}} \]

\[ \text{GFP}_{\text{moPrP}} \]

\[ \text{GFP}_{\text{moPrP}^*} \]

\[ \text{MVKSHIGSWILVLFAVNSDVGLC} \]

\[ \text{MANLYWLLALFVTDTVGLC} \]

\[ \text{gtc atc ATG gtg} \]

\[ \text{gtc atc ATG gcg} \]

\[ \text{agt tct ATG gcg} \]

\[ \text{GFP} \]

\[ \text{GFP} \]

\[ \text{GFP} \]
Figure 6C

- GFPmoPrP
- GFPmoPrP*

Full length

Ab GFP

kDa

60 -
50 -
40 -

1 2

Full length GFPmoPrP
Figure 7

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kDa 40- 30- 20-  

Ab P4 Ab P4

Full length PrP

N-terminal fragment
Alternative translation initiation generates cytoplasmic sheep prion protein
Christoffer Lund, Christel M. Olsen, Susan Skogtvedt, Heidi Tveit, Kristian Prydz and
Michael A. Tranulis

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