neurodegenerative diseases through its conversion into self-perpetuating and neurotoxic forms (1–4). Despite a large amount of evidence supporting a role in survival/death and growth/differentiation cell decisions, the physiological function of PrPC and its involvement in disease remains elusive (5–8). A crucial limiting factor for PrPC functional determination is its molecular diversity. Although PrPC is mainly thought of as a glycoprotein attached to the cell surface by a glycosylphosphatidylinositol anchor, PrPC is actually synthesized as a family of four members: the membrane anchored glycoprotein (NterminusPrP), two transmembrane forms with opposite topologies (NterminusPrP and CterminusPrP), and a soluble form (CysPrP) (3, 9–12).

This article has been withdrawn by the authors. We have become aware of errors in the preparation of Figs. 2 and 3, where some lanes appear duplicated. Although replicated experiments performed at the time support the results and conclusions presented in the published paper, we believe that the responsible course of action is to withdraw the article in the interests of maintaining the publication standards of the journal. We apologize for any inconvenience we may have caused. The paper with the corrected figures can be obtained by contacting the authors.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Recombinant Standard Production—The plasmid pDNA4-HaPrP, kindly provided by Dr. R. S. Hegde, was first mutatated to introduce the six nucleotides from the 5′-untranslated region adjacent to the initial ATG to preserve the wild type Kozak sequence. The HuPrP open reading frame was cloned into pcDNA3.1 at BamHI/EcoRI sites preserving the corresponding wild type Kozak region. Wild type constructs were used as templates to generate different mutants (see Table 1 and Fig. 1) by using the Quikchange protocols (Stratagene). The integrity of each construct was verified by sequencing. Recombinant PrP chains of 1–254, 15–231, 1–254, and 15–254, and 23–231 were produced from the corresponding pET11a plasmids in Escherichia coli BL21(DE3) and used as inclusion bodies denatured extracts as described previously (5).

Transcription, Translation, and Translocation Assays—All plasmids were enzymatically linearized (pDNA4.1-HaPrP plasmids with ApaI and pDNA3.1-HuPrP constructs with SacII) and then transcribed with the T7 CapScribe kit (Promega). After integrity verification, the transcribed mRNAs
were translated at 80 μg/ml final concentration using the 50% (v/v) nuclease-treated rabbit reticulocyte lysate system (Promega) and Redivue™ 1-[35S]methionine (Amersham Biosciences), as indicated by the manufacturer. For translation-translocation assays, the reaction mixture was enriched in 15% (v/v) canine pancreatic rough microsomal membranes (Ref. 24 and references therein). Isolation of the fraction of sealed microsomes from the reaction mixtures was performed by discontinuous sucrose gradient ultracentrifugation as described previously (24). For protease protection analysis, the total reaction mixtures and their sealed microsomal fractions were incubated for 1 h at 4 °C with 0.1 mg/ml proteinase K (Roche Diagnostics) both in the absence and in the presence of 0.5% Triton X-100. The reaction was stopped with 5 mM phenylmethylsulfonyl fluoride. The 35S-labeled reaction products were immunoprecipitated with αPrP 3F4 monoclonal antibody (Signet Laboratories), resolved on Tris-Tricine 16.5% SDS-PAGE gels, and visualized using a phosphorimaging device (Fuji FLA-3000). Enzymatic deglycosylation was performed by incubating the immunoprecipitated samples with PNGase F (New England Biolabs) according to the manufacturer’s instructions.

**Cell Culture, Transfections, and Treatments**—CHO and COS-7 cells were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 IU/ml penicillin, and 10 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. Transfection of cells with the different plasmids was performed with TransIT-LT1 (Mirus) following the manufacturer’s instructions. After 48 h, cells were processed for bulk selection of stable transfectants. For immunofluorescence and confocal microscopy experiments, 24 h after transfection, the medium was changed, and the incubation was continued for another 14 h. Cells were harvested and analyzed for PrP expression (see below).

**Cell Lysates, Brain Homogenates, and Immunoprecipitations**—Denatured cell lysates were prepared at about 15 mg/ml protein concentration in 62.5 mM Tris-HCl, pH 6.8, containing 4% (w/v) SDS and 25% (v/v) glycerol. After a 10-min spin at 10,000 × g, the supernatants were diluted 1:40 with PBS, pH 7.4, containing 0.1% sodium deoxycholate (Calbiochem), 1% Nonidet P-40 (Sigma-Aldrich), 1.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 1 h with protein A/G-Sepharose (Amersham Biosciences). After a 5-min centrifugation at 10,000 × g, the supernatants were incubated with protein G-Sepharose conjugated with PrP antibodies at 4 °C, and the resulting pellets were captured with protein A/G-Sepharose and eluted in 2X SDS-PAGE sample buffer for isolation of the nuclear fractions. The postnuclear supernatants were eluted in 62.5 mM Tris-HCl, pH 8.0, 1% SDS, and equal aliquots of each fraction were analyzed by SDS-PAGE and immunoblotting.

**Immunoprecipitation and Western Blotting**—Samples were lysed in 62.5 mM Tris-HCl, pH 6.8, containing 4% (w/v) SDS, and 25% (w/v) glycerol. After a 10-min spin at 10,000 × g, the supernatants were diluted 1:40 with PBS, pH 7.4, containing 0.1% sodium deoxycholate (Calbiochem), 1% Nonidet P-40 (Sigma-Aldrich), 1.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 1 h with protein A/G-Sepharose (Amersham Biosciences). After a 5-min centrifugation at 10,000 × g, the supernatants were incubated with protein G-Sepharose conjugated with PrP antibodies at 4 °C, and the resulting pellets were captured with protein A/G-Sepharose and eluted in 2X SDS-PAGE sample buffer for isolation of the nuclear fractions. The postnuclear supernatants were eluted in 62.5 mM Tris-HCl, pH 8.0, 1% SDS, and equal aliquots of each fraction were analyzed by SDS-PAGE and immunoblotting.

**Protein Aggregation Assays**—Analysis of PrP aggregation upon proteasome inhibition was performed with minor modifications to published methods (13, 15–17). Cells were lysed in cold EZ-lysis buffer and then separated into pellet (nuclear) and supernatant (postnuclear) fractions by a 500 × g centrifugation for 10 min at 4 °C. The pellets were washed twice with EZ-lysis buffer for isolation of the nuclear fractions. The postnuclear supernatants were supplemented with 0.5% Triton X-100 and 0.5% deoxycholate, dispersed by extensive pipetting, and then centrifuged for 10 min at 13,000 × g at 4 °C. Proteins in the supernatant were precipitated with cold 15% trichloroacetic acid. All protein pellets were resuspended in 0.1 mM Tris-HCl, pH 8.0, 1% SDS, and equal aliquots of each fraction were analyzed by SDS-PAGE and immunoblotting.

**Confocal Fluorescence Microscopy**—Cells were plated onto glass coverslips, allowed to attach for 24 h, and then transfected for 48 h. Cells were fixed with 4% paraformaldehyde in PBS containing 5% sucrose for 10 min at room temperature and washed three times with PBS. Cells were permeabilized and blocked in PBS containing 0.5% saponin, 0.1% Triton, and 2% bovine serum albumin for 10 min at room temperature. After fixation, cells were washed with blocking buffer, and samples were incubated with Alexa Fluor-647-conjugated goat α-mouse IgG (1:800), Alexa Fluor-488-conjugated α-rabbit IgG (1:800), and Hoechst 33342 (10 μg/ml) in blocking solution for 30 min at room temperature. After washing, the coverslips were mounted on glass slides with ProLong Gold antifade reagent (Molecular Probes). Images were captured using a confocal microscope (Leica TCS-SP-AOBS-UV) using
the UV and argon lasers at 20 milliwatt for excitations at 364 nm (Hoescht) and 488 nm (Alexa Fluor-488), respectively, and the 633-nm line of the He-Ne laser at 10 milliwatt for excitation at 647 nm. Image analysis was performed using Leica confocal software.

Cell Proliferation Assays and Cell Cycle Analysis—For cell growth analysis, cells were co-transfected with pEYFP (Clontech) and the plasmid coding wild type HaPrP or its mutants. After 48 h of transfection, cells were synchronized in serum deprivation for 18 h and then released by serum supplementation for 6 h. Cell proliferation was analyzed in the 96-well plate format using the bromodeoxyuridine cell proliferation kit (Calbiochem) and a MR500 microplate reader (Dynatech). Bromodeoxyuridine labeling was divided in three aliquots: (i) untreated, (ii) digested with proteinase K, and (iii) treated with PNGase F as indicated. Relative sample loads are indicated at the bottom of each panel. Accessibility of the translation-translocation products of each of the mRNAs was translated in the rabbit reticulocyte lysate translation system in the presence of pancreatic microsomes and then digested with proteinase K (PK) in the presence of 0.5% Triton X-100 (Tx) as a control for full accessibility. For SM analysis, HaPrP(M1S) was loaded in a 10× excess as compared with HaPrP WT.

RESULTS

N-terminal Signal Peptides of PrP Contain a Dual Methionine Motif—N-terminal signal peptides display a tripartite organization into n-, h-, and c-regions, with the hydrophobic central region (h-region) essential for co-translation membrane integration and translocation process. The signal sequences of PrP from different species can be classified into three groups on the basis of the number of Met residues and their position with respect to regional boundaries (Fig. 1). Group I, represented by the rodent sequences, contains two Met residues at positions 1 and 15; the second position is in the N-terminal side of the c-region. In Group II, represented by the human sequence, the two Met residues are at positions 1 and 8. In this case, the second Met constitutes the N terminus of the h-region. On the contrary, Group III, which is represented by the mink sequence, lacks the second Met residue. When converted into their cognate mRNA sequences, the Met residues of the signal sequences become AUG codons that could behave as translation initiation sites. We also identified two in-frame triplets (CUG and GUG coding HaPrP L9 and V13) that could sustain translation initiation by means of a single base difference. These non-AUG codons are conserved in all species. If used, any of these codons could yield nascent chains with different cellular fates.

The MM Motif Allows a Dual Translation Start and the Existence of PrP Isoforms—To test whether the downstream AUG codons found in the signal sequence regions of PrP mRNA in Group I and Group II could sustain translation initiation, we generated a series of point mutations in both the HaPrP and the HuPrP open reading frames (Figs. 1 and 2 and Table 1). These mutations consisted of the insertion of a C or a G at various positions causing a +1 shift in the reading frame (11C12, 13G14, 16C17), as well as an Met-to-Ser substitution (ATG-to-TTC). The reading frameshift mutations allow the study of both non-AUG and AUG start sites, whereas the Met-to-Ser substitutions permit the evaluation of the role of a specific Met residue. It should be noted that frameshift mutations allow translation initiation at either start site, but only the product produced from the start site downstream from the insertion will proceed to the wild type (WT) stop codon and will produce chains retaining the 3F4 PrP epitope.
Nucleocyttoplasmic PrP isoforms

TABLE 1
HaPrP and HuPrP constructs
The HaPrP open reading frame, cloned into pcDNA4.1 under BglII/EcoRI targets, and the HuPrP open reading frame, cloned into pcDNA3.1 under BamHI/EcoRI targets, were used as templates for the generation of point, reading shift, and deletion mutants using standard molecular biology protocols.

| Name              | Mutation                  | Forward oligonucleotide |
|-------------------|---------------------------|-------------------------|
| HaPrPwt           |                           |                         |
| HaPrP(M1S)        | ATG-1-TTC                 | 5'-GATCTACCTCGGACCTACAGC-3' |
| HaPrP(M15S)       | ATG-15-TTC                | 5'-CTTTCTGGAGGTAGCTGAGATGTTGG-3' |
| HaPrP(M15S,M15S)  | ATG-1-TTC                 | 5'-CTTTCTGGAGGTAGCTGAGATGTTGG-3' |
| HaPrP(11C12)      | +1 shift downstream codon 11 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HaPrP(13G14)      | +1 shift downstream codon 13 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HaPrP(16C17)      | +1 shift downstream codon 16 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HaPrP(D14)        | Deletion of the 1–14 region | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HuPrP WT          |                           |                         |
| HuPrP(M1S)        | ATG-1-TTC                 | 5'-GATCGAGATCCGACCTGACATGGATACCACTGG-3' |
| HuPrP(M8S)        | ATG-8-TTC                 | 5'-CTTTCTGGAGGTAGCTGAGATGTTGG-3' |
| HuPrP(M15S,M15S)  | ATG-15-TTC                | 5'-CTTTCTGGAGGTAGCTGAGATGTTGG-3' |
| HuPrP(5C6)        | +1 shift downstream codon 5 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HuPrP(11C12)      | +1 shift downstream codon 11 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HuPrP(13C14)      | +1 shift downstream codon 13 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |
| HuPrP(16C17)      | +1 shift downstream codon 16 | 5'-GCTGGATGCTGGTTCCTGGTTCTCTCCGGTATG-3' |

FIGURE 3. Expression of HaPrP(M15) in CHO cells. A, transient expression of HaPrP WT and of its mutants in CHO cells. After 48 h of co-transfection with pEYFP, cells were harvested, lysed, and immunoblotted with antibodies against PrP (3F4), YFP, and β-actin, respectively. Similar results were obtained using COS-7 cells. B, comparison of HaPrP(M15S) with 1–254, 15–254, 15–231, and 23–231 PrP chains produced in E. coli inclusion bodies by Western blot. The Tris-Tricine SDS-PAGE was performed using 16.5% gels, and the blot was probed with 3F4. The migration of the recombinant PrP-(15–254) chain is depicted with a straight line. C, enzymatic deglycosylation with PNGase F of HaPrP WT and of its M15 mutant. D, incorporation of HaPrP WT and of its M15 and Δ14 mutants into cytosolic insoluble aggregates upon proteasome inhibition. After transfection (30 h), cells were treated in the absence or presence of 5 μM MG132. Incubation with MG132 was allowed to proceed for either 24 h (+24) (irreversible inhibition) or after 4 h (+4), the medium were replaced with MG132-free medium, and the incubation was continued for other 16 h (transient inhibition). Insoluble cytosolic aggregates were isolated as described under “Experimental Procedures.”

The results of the translation of the mRNAs coding for WT and mutant HaPrP and HuPrP using reticulocyte lysates followed by immunoprecipitation with 3F4 are shown in Fig. 2A. In agreement with previous reports, wild type HaPrP mRNA was translated into a major polypeptide chain of about 26 kDa. Under the same conditions, translation of the mRNA coding HaPrP(M15S), in which the canonical AUG is functionally impaired, and for the reading frameshift mutants HaPrP(11C12) and HaPrP(13G14) led to the production of a single product of similar mass (26 kDa) but reduced intensity (about 15% of that of the WT). On the contrary, translation of the mRNAs HaPrP(M16C17) and HuPrP(M15S,M15S) resulted in the absence of any detectable signal. These results support the idea that HaPrP mRNA contains a minor translation initiation site and that this site is located at codon 15, the AUG triplet coding for Met-15. It should be noted that the chains translated from Met-1 and Met-15 could not be easily differentiated by electrophoresis, probably as a result of the balance between the differences in size and hydrophobicity of the chains (25).

The translation of WT HuPrP mRNA yielded a band corresponding to a polypeptide chain of about 27 kDa (Fig. 2A). This band was detected using the mRNAs of the HuPrP(M15S) and HuPrP(5C6) mutants but with less intensity. On the contrary, this band was not observed using the mRNAs of the HuPrP(M15S,M15S) and HuPrP(M15S,M15S) mutants. These results show that HuPrP mRNA, as model for Group II, also contains a minor translation start site and that this site is located at codon 8, the AUG triplet coding for Met-8. Because the alternative translation start site of PrP signal sequences in groups I and II is due to the dual methionine motif, it follows that the sequences of Group III either lack this capacity or utilize a different process.
HaPrP(M15) and HuPrP(M8) Isoforms

Synthesized CysPrP—To unambiguously establish the relationship between HaPrP(M15) and HuPrP(M15), we studied their behavior in cell-free biosynthesis assays (Fig. 2, B and C). Fig. 2B shows that the products of mRNAs, the products of mRNAs coding HaPrP(WT) and HuPrP(M15) mutants and translated in the presence of microsomal membranes consisted of a single band of 26 kDa that remained unchanged after PNGase F digestion. Comparison of the bands after deglycosilation, in particular of HuPrP chains, suggests that HuPrP(M1S) migrates similarly to an unprocessed full-length chain (see below) (26). The unglycosylated pattern agrees with a cytosolic location for the HaPrP(M15) and HuPrP(M8) C-terminal domains. Furthermore, external addition of proteinase K to both the total reaction mixture and its sealed microsome fraction (no signal was detected in this fraction even using a 10× overload as compared with the WT) resulted in complete degradation of the ∼26-kDa chains translated from the HaPrP(M15) and HuPrP(M8) mRNAs (Fig. 2C). In contrast, the product translated from WT mRNAs under similar conditions showed protected fragments corresponding to translocated and integrated PrP chains (3). Taken together, these results suggest that HaPrP(M15) and HuPrP(M8) chains segregate outside the secretary route under a proteinase K-sensitive conformation as described for CysPrP.

To determine whether the synthesis of these isoforms takes place in cellular contexts, we proceeded with transient transfection experiments using CHO and COS-7 cells, which have undetectable levels of endogenous PrP expression. In this case, the study was restricted to the HaPrP sequences for biosafety reasons and was performed as co-transfection with pEYFP to use glycosylation as an internal control. Plasmids encoding WT HaPrP and HuPrP(M1S/M15S) were used as positive controls for PrP expression, respectively, whereas those encoding HaPrP(11C12) and HaPrP(M15) were employed to assess the functionality of M15 as start site. Fig. 3A shows that HaPrP(M15) was indeed synthesized by cells based on the presence of a 26-kDa band recognized by α-PrP 3F4 in the lysates of HaPrP(M11C12) and HaPrP(M15) transfectants. Importantly, the 26-kDa band was also recognized by α-SP, an antibody raised against the C-terminal region of the signal sequence (11). In cell lysates, HaPrP(M15) retained the C-terminal hydrophobic segment according to electrophoretic mobility determinations using a panel of recombinant PrP chains consisting of the full unprocessed chain (1–254 sequence), the fully processed chain (23–231 sequence), and N-terminally shortened chains either containing (15–254) or lacking (15–231) the C-terminal hydrophobic segment (Fig. 3B).

To corroborate that the HaPrP(M15) synthesized in cells behaves as CysPrP, we studied its glycosylation state as well as its capacity to form insoluble aggregates upon proteasome impairment (13, 15–17). Fig. 3C shows that in contrast to WT HaPrP, 26-kDa HaPrP(M15S) remained unchanged upon PNGase F digestion as expected for CysPrP. Moreover, both transient and irreversible inhibition of the proteasome with 5 μM MG132 promoted the formation in the cytosol of insoluble HaPrP(M15S) aggregates (Fig. 3D). Both the absence of glycosylation and the capacity to form cytosolic insoluble aggregates confirms that HaPrP(M15) behaves as CysPrP in a cellular context.
Nucleocytoplasmic PrP isoforms

HaPrP(M15) and HuPrP(M8) Are Found in Nuclei Isolated from Cells and Normal Brain Homogenates and Are Sumoylated—To elucidate the properties of these isoforms, we first studied their subcellular location using confocal microscopy. Unless stated, HaPrP(M15) was expressed from the HaPrP(A14) construct for easier detection. Indirect immunofluorescence stainings showed that at 48 h after transfection, HaPrP(M15) was localized largely to the nuclei of cells (Fig. 4A).

The distribution pattern agreed with the diffuse nucleoplasmic location observed for several studied PrP models (19, 27) and differed from the intranuclear granules observed in neuronal cells expressing bovine PrP<sup>C</sup> (28). The cellular localization was then confirmed by subcellular fractionation of cell homogenates. Fig. 4B shows that about 70% of the expressed HaPrP(A14) was localized to the nuclear fraction, mainly as a 26-kDa chain but also as higher molecular weight species. Similar results were obtained using CHO and COS-7 cells.

To generalize the nuclear localization of HaPrP(M15), as well as to determine the origin of the high molecular weight bands, we purified the nuclei from normal hamster brain and human cortex homogenates and characterized the PrP contained therein. Before the analysis, the purified nuclei were dispersed in EZ-lysis buffer, digested with PIPLC, and then centrifuged at low speed. This process allows the release of the contaminant membrane-anchored forms (29). Fig. 5A shows that after removing raft-resident PrP<sup>C</sup>, PrP was detected in the nuclei purified from hamster brain homogenates as two bands of 26 and 35 kDa that remained unchanged after enzymatic deglycosylation. Nuclear PrP in human cortex was also comprised of two major PNGase F-resistant bands of about 26 and 35 kDa. These bands were recognized by both 3F4 and SP, as expected from PrP chains bearing N-terminal shortened signal peptides (Fig. 5B). These data confirm the existence and nuclear accumulation of isoforms produced by alternative translation in normal tissues.

The complexity of the bands suggests the occurrence of covalent modifications. Of the modifications that can occur in nuclear proteins and cause increases in size, activity-modifying sumoylation and degradation-targeting ubiquitylation were studied. Fig. 5C shows that high molecular weight bands of PrP immunoprecipitated with 3F4 from denatured nuclei extracts of hamster brain homogenates were recognized by an anti-SUMO-1 antibody but not by anti-ubiquitin or anti-SUMO 2/3 antibodies. Inverse pull-down experiments with α-SUMO-1 confirmed 3F4 immunoreactivity. Because SUMO-1 conjugation involves the covalent attachment of a single 9.5-kDa chain, the observed band pattern can be explained to a large extent by considering the composition of a non-sumoylated chain (26 kDa) and a SUMO-1-conjugated form (35 kDa). In summary, PrP(M8/M15) appear to be a nuclear isoform that acts as substrate for SUMO-1 conjugation.

HaPrP(M15) Expression Abrogates Cell Proliferation—Trials to establish cell lines expressing HaPrP(M15) were unsuccessful despite the absence of a conclusive and reproducible cell death event. Stably transfected clones were selected, but they failed to grow. These growth alterations together with the nuclear distribution and involvement of reversible sumoylation prompted us to consider a possible antiproliferative activity.

Analysis of bromodeoxyuridine incorporation showed that HaPrP(M15) did indeed decrease cell growth as compared with HaPrP<sup>WT</sup> and the negative control HaPrP(M1SM15S) in both COS-7 and CHO cells (Fig. 6A). This effect was more pro-

FIGURE 6. HaPrP(M15) expression interferes with cell growth. A, the expression levels of PrP<sup>C</sup> mutants on CHO and COS7 cell proliferation measured by cell sorting. Data are shown as the mean ± S.D. of three independent experiments performed in triplicate. Results were compared by one-way analysis of variance with Bonferroni’s post-test analysis using GraphPad Prism, version 4.0. Statistically significant differences between groups are indicated (***, p < 0.001). For both panels, A and B, the relative expression level of the constructs was indicated (***, p < 0.001). B, experiment was performed in duplicate. Data were compared by two-way analysis of variance with Bonferroni’s post-test analysis using GraphPad Prism, version 4.0. Statistically significant differences between groups are indicated (***, p < 0.001). For both panels, A and B, the relative expression level of the constructs was indicated (***, p < 0.001).
nounced and statistically significantly higher \((p < 0.001)\) for the HaPrP(Δ14) mutant, which overexpresses the PrP isoform (Fig. 3A), than for the HaPrP(M1S) and HaPrP(11C12) mutants.

The cell cycle was then analyzed using a co-transfection approach. In this case, cells were co-transfected with pEYFP for separation of the positive transfectants by cell sorting before propidium iodide labeling. Fig. 6, B and C, shows that cells expressing HaPrP(M15) from both HaPrP(Δ14) and HaPrP(11C12) constructs exhibited a higher proportion of cells in the \(G_0/G_1\) phase as compared with the mock control (transfection with HaPrP(M1SM15S)). These results indicate that HaPrP(M15) functions as a growth suppressor that delays the exit from \(G_1\) phase.

DISCUSSION

In this study, we have shown that the minor member of the PrP\(^{C}\) family segregating outside the secretory route is generated by alternative initiation of translation. The presence of a second Met residue at the h-region boundary of the signal sequence determines the alternative translation initiation event. This process permits the synthesis of an isoform translated either from Met-15 in HaPrP or from Met-8 in HuPrP. This isoform represents a novel chain differing from the conventional mature form in the retention of the c-region of the N-terminal signal sequence and the full C-terminal hydrophobic region. These two segments could provide new functions as stability regulators or as sites of interaction for distinct ligands, among others.

The use of in-frame alternative translation is a relatively common process by which a single mRNA can acquire a multiplicity of sorting and function under environmental regulation (30–34). The relative common process by which proteins encoded by a single mRNA can acquire a multiplicity of sorting and function under environmental regulation (30–34). Although the relative proportion of PrP(M15) synthesized in vitro studies and in the cell system used here is very low, it might be susceptible to such modulation. This is supported by the cell and regional dependence of PrP expression in normal rodent brains as well as its increased levels under endoplasmic reticulum stress (23, 35). As noted, the dual start site motif is missing in a group of highly conserved PrP sequences. In these sequences, the AUG triplet coding for Met-15 is found as either ACG or ACA, which code for Thr. Of these two codons, ACG can function as a non-AUG start site (36, 37). However, the function of the ACA triplet as a start is unclear; thus whether ACA-bearing species use the alternative translation mechanism remains to be established.

Isolating the synthesis of HaPrP(M15) from that of the major membrane-bound PrP forms allowed three major findings: nuclear localization, variable SUMO-1 conjugation, and antiproliferative activity. The nuclear localization of this isoform might explain previous PrP studies describing rare nuclear localization, the presence of nuclear localization sequence, and the capacity of the chain to interact with nucleic acids and with chromatin (19, 28, 38).

SUMO-1 conjugation of the nuclear population of PrP suggests stringent regulation of activity and physiological relevance for this isoform. In general, sumoylation provides an on/off functional switch for protein interactions involved in processes such as transport, transcriptional silencing, genomic stabilization, and stress responses (39). SUMO-1-conjugated and free HaPrP(M15) chains might thus represent alternative functional states of the molecule. It is thus interesting to note that the degree of sumoylation in nuclei from brains was higher than that in cells. With the limitations imposed by the lack of sumoylation control, HaPrP(M15) expression might be involved in dysregulation of cellular growth resulting in \(G_0/G_1\) phase arrest.

The antiproliferative function of HaPrP(M15) expands the physiological role of PrP\(^{C}\). Because most cells withdraw from the cell cycle to differentiate during the \(G_1\) phase, it is tempting to consider HaPrP(M15) as candidate for promotion of \(G_1\) phase arrest required for cell differentiation in some developing tissues (7). On the other hand, the loss of HaPrP(M15) nuclear functionality might favor either cell transformation on depletion (8) or cell death on cytosolic accumulation (15). HaPrP(M15) can regulate the efficiency of prion accumulation, which decreases with cell division (40). The isolation of the synthesis of this isoform from that of other members of the PrP\(^{C}\) family suggests that each member of this family might have different physiological roles and that their aberrant cross-talk could account for the pathogenic mechanism.

Acknowledgments—We thank C. Albo and M. Seisdedos for technical assistance, S. Hegde, C. Korth, J. Fornaro, and E. Zavala for sharing reagents and for helpful discussions.
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October 27, 2017
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J. Biol. Chem. 2009, 284:2787-2794.
doi: 10.1074/jbc.M804051200 originally published online December 5, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M804051200

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October 27, 2017