

An overlapping reading frame in the *PRNP* gene encodes a novel polypeptide distinct from the prion protein

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ABSTRACT The prion protein gene *PRNP* directs the synthesis of one of the most intensively studied mammalian proteins, the prion protein (PrP). Yet the physiological function of PrP has remained elusive and has created controversies in the literature. We found a downstream alternative translation initiation AUG codon surrounded by an optimal Kozak sequence in the +3 reading frame of *PRNP*. The corresponding alternative open reading frame encodes a polypeptide termed alternative prion protein (AltPrP) with a completely different amino acid sequence from PrP. We introduced a hemagglutinin (HA) tag in frame with AltPrP in PrP cDNAs from different species to test the expression of this novel polypeptide using anti-HA antibodies. AltPrP is constitutively coexpressed with human, bovine, sheep, and deer PrP. AltPrP is localized at the mitochondria and is up-regulated by endoplasmic reticulum stress and proteasomal inhibition. Generation of anti-AltPrP antibodies allowed us to test for endogenous expression of AltPrP in wild-type human cells expressing PrP. By transfecting cells with siRNA against PrP mRNA, we repressed expression of both PrP and AltPrP, confirming endogenous expression of AltPrP from *PRNP*. AltPrP was also detected in human brain homogenate, primary neurons, and peripheral blood mononuclear cells. These results demonstrate an unexpected function for *PRNP*, which, in addition to plasma membrane-anchored PrP, also encodes a second polypeptide termed AltPrP.—Vanderperre, B., Staskevicius, A. B., Tremblay, G., McCoy, M., O'Neill, M. A., Cashman, N. R., Roucou, X. An overlapping reading frame in the *PRNP* gene encodes a novel polypeptide distinct from the prion protein. *FASEB J.* 25, 2373–2386 (2011). www.fasebj.org

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THE PRION PROTEIN (PrP) IS A glycoprotein anchored to the plasma membrane by virtue of a glycosylphosphatidylinositol (GPI) anchor (1). Transmissible spongiform encephalopathies (TSEs) involve the conversion

of PrP^C, the normally folded conformer of PrP, into PrP^{Sc}, an aggregation-prone isoform of PrP that is resistant to proteinase K (2). PrP^{Sc} is known to be the infectious agent in TSEs. However, the presence of normally folded PrP^C is absolutely necessary for the onset of disease, as it acts as a continuous supply for the generation of PrP^{Sc}. Therefore, *PRNP*, the gene encoding PrP, is essential for the development of TSEs, and not surprisingly, PrP knockout animals are resistant to prion infection (3–5). Many missense mutations within human *PRNP* are also associated with genetic forms of TSEs (6), providing support for a central role of PrP in TSE pathogenesis.

In contrast to its well-established pathogenic role, the quest for the normal physiological function of PrP has proven very difficult. Several functions have been proposed, and controversies on the role of PrP continue in the literature (7). Some of the explanations put forward include the study of different cultured cell models used to investigate the function of PrP, such as neuronal *vs.* non-neuronal cell lines, immortalized cell lines *vs.* primary cells, and mouse neurons *vs.* human neurons. How one gene and the associated protein can result in such a complexity in terms of physiological function is confounding.

It has recently been hypothesized that alternative translation initiation of eukaryotic mRNAs might be used as a method to expand the proteome (8). On the basis of the idea that a single mRNA can produce 3 completely independent amino acid sequences if read in all 3 possible reading frames, this hypothesis suggests that the complexity of the eukaryotic proteome is largely underestimated. Several examples of out-of-frame alternative translation initiation in eukaryotes exist to support this hypothesis. However, almost all of these examples occur at an upstream AUG codon in

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relation to the +1 position of the main open reading frame (ORF) (9, 10). These alternative AUG codons are usually situated within an optimal Kozak context (11). Although extremely rare, a small number of examples of out-of-frame alternative translation initiation at a downstream AUG codon in an optimal Kozak context exist in mammals (12, 13).

On reexamination of the PrP-coding sequence (CDS), we found a potential ORF whose initiator codon is surrounded by an optimal Kozak context in a number of species. In this study, we show that the protein encoded in this overlapping ORF is coexpressed with PrP from the *PRNP* gene. This finding has direct implications regarding the comprehension of the physiological function of *PRNP*.

MATERIALS AND METHODS

Cloning of plasmids and transfection

All primer sequences are outlined in **Table 1**. Cloning of human PrP^C in pCEP4 β vector (Invitrogen, Carlsbad, CA, USA) has been described previously (14). Human PrP^(HA) was produced by inserting an HA tag in the +3 frame of human PrP between bases 308 and 309 of the PrP CDS by PCR overlap extension using forward primers 1 and 3 and reverse primers 2 and 4. Human PrP^{(HA)*}, in which the alternative AUG at bp 90–92 of PrP^(HA) was mutated to CUG, was produced by the Quikchange method (Stratagene, La

Jolla, CA, USA) using forward primer 5 and reverse primer 6. Human C-terminally tagged AltPrP^{HA} was produced using huPrP^(HA) as a template with forward primer 7 and reverse primer 9. Untagged human AltPrP was amplified from huPrP^C with forward primer 7 and reverse primer 8. Human PrP Δ 1–66(HA), in which the first 66 bp of the PrP^(HA) CDS were deleted, was produced using forward primer 10 and reverse primer 2. All constructs were inserted in pCEP4 β vector using *Hind*III and *Bam*HI restriction enzymes. Recombinant huAltPrP was produced with forward primer 11 and reverse primer 12. The PCR product was then inserted in pET-21b vector (EMD Chemicals, Gibbstown, NJ, USA) using *Nhe*I and *Bam*HI restriction enzymes. The recombinant protein was then expressed as described previously (15). Bovine PRNP was reverse-transcribed from a total RNA extract using forward primer 13 and reverse primer 14. The bovine PrP CDS was then amplified using forward primer 15 and reverse primer 16. Bovine PrP was then inserted in pCEP4 β vector using *Hind*III and *Not*I restriction enzymes. Bovine PrP^(HA) and AltPrP^{HA} were produced as described above with primers 15, 16, 17, 18, 19, and 20, and inserted in pCEP4 β vector using *Hind*III and *Bam*HI restriction enzymes. White-tailed deer (wt) PrP in pCEP4 β was a kind gift from Debbie McKenzie (Alberta Centre for Prion and Protein Folding Diseases, Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada). wtdPrP^(HA) and AltPrP^{HA} were produced as described above with primers 15, 16, 19, 20, 21, and 22. PCR products were then inserted in pCEP4 β vector using *Hind*III and *Bam*HI restriction enzymes. Sheep PrP in pCI expression vector was a kind gift from Dr. Michael A. Tranulis (Norwegian School of Veterinary Science, Dept. of Biochemistry and Physiology, Institute of Basic Sciences and Aquatic Medicine, Oslo, Norway). Sheep PrP^(HA) and

TABLE 1. *Primer sequences*

Primer	Sequence, 5'–3'
huPrP F	CCCAAGCTTGTAAATGGCGAACCTTGGCTGCTGG
huPrP R	CGCGGATCCTCATCCACTATCAGGAAGATG
huPrP ^(HA) F	TATCCGTACGACGTACCAGACTACGCCTAAGCCAAAAACCAAC
huPrP ^(HA) R	GTGGAACAAGCCGAGTATCCGTACGACGTACCAGACTACGCC
huPrP ^{(HA)*} F	CGAAGCCTGGAGGCTGGAACACTGGGG
huPrP ^{(HA)*} R	CCCCAGTGTTCAGCCTCCAGGCTTCG
huAltPrP F	GGGAAGCTTGCCATGGAACCTCTGGGGGCA
huAltPrP R	CCCGGATCCTTACTCGGCTTGTTTC
huAltPrP ^{HA} R	CGACGTACCAGACTACGCCTAAGGATCCGA
hu Δ 1–66 F	AGTAAGCTTGCCGCCATGGGCCTCTGCAAGAAGCGCCCGAAGC
huAltPrP pET F	AGGCTAGCGAACACTGGGGGCGAGCCGATA
huAltPrP pET R	TCCGATCCTTACTCGGCTTGTTCCACTGACTGT
boPrP F	TTCAACCAAGCCGAAGCATCTGTC
boPrP R	AGCACGAAATGAGACACCACCACT
boPrP ^(HA) F	TATCCGTACGACGTACCAGACTACGCCTAAGCCAAAAACCAAC
boPrP ^(HA) R	GGCGTAGTCTGGTACGTCTACGGATACTCTGGGTTTGTTC
boAltPrP F	ATCAAGCTTAGGAGGATGGAACACTGG
boAltPrP ^{HA} R	GATGGATCCTTAGGCGTAGTCTGGTAC
wtdPrP F	ACACCTCTTTATTTTGCAG
wtdPrP R	AGAAGATAATGAAAACAGGAAGG
wtdPrP ^(HA) F	TATCCGTACGACGTACCAGACTACGCCTAAGCCAAAAACCAAC
wtdPrP ^(HA) R	GGCGTAGTCTGGTACGTCTACGGATACTCTGGGCTTGTTC
shPrP F	TAGCCTCGAGGTCATCATGGTGAAAAGCCACATAGG
shPrP R	CGACTCTAGAGTACTATCTACTATGAGAAAAATGAGG
shPrP ^(HA) F	TATCCGTACGACGTACCAGACTACGCCTAGCCACAGTCAGTGAACAAG
shPrP ^(HA) R	GGCGTAGTCTGGTACGTCTACGGATACTCCACCTTGACCCAG
shAltPrP ^{HA} F	TATCTCGAGGCCACCATGGAACACTGGGGGGAGCC
shAltPrP ^{HA} R	GGCTGGGGTCAAGGTGGGAGTATCCGTACGACGTACCAGACTACGCCTAGTCTAGATAT

F, forward; R, reverse.

AltPrP^{HA} were produced as described above with primers 23, 24, 25, 26, 27, and 28. PCR products were then reinserted in the pCI expression vector (Promega, Madison, WI, USA) using *XhoI* and *XbaI* restriction enzymes. All restriction enzymes were acquired from New England Biolabs (Ipswich, MA, USA). Cells were transfected with ExGen 500 transfection reagent (Fermentas, Burlington, ON, Canada) or GeneCellin transfection reagent (BioCellChallenge, Toulon, France), according to the manufacturer's instructions.

Antibodies and reagents

Primary antibodies used were monoclonal anti-Cox IV (ab14744), polyclonal anti-VDAC1 (ab15895), polyclonal anti-GRP78 BiP (ab53068), polyclonal anti-GAPDH (ab9485), polyclonal anti-HA (ab9110), and monoclonal anti-phospho-eIF2 α (ab32157; Abcam, Cambridge, UK); monoclonal anti- β -actin (clone AC-15; Sigma-Aldrich, St. Louis, MO, USA); monoclonal anti-Hsp70 (SPA-810, Stressgen, Ann Arbor, MI, USA); polyclonal anti-Bax (sc-493; Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-cytochrome *c* (clone 6H2.B4; BD Pharmingen, Franklin Lakes, NJ, USA); monoclonal anti-HA (clone C29F4; Cell Signaling Technology, Danvers, MA, USA); monoclonal anti-mitochondrial Hsp70 (clone JG1; Affinity BioReagents, Waltham, MA, USA); monoclonal anti-PrP (clone SAF32; Cayman Chemical, Ann Arbor, MI, USA); and monoclonal anti- α -tubulin (clone A11126; Molecular Probes, Eugene, OR, USA). Anti-PrP clone 3F4 was purified from hybridoma cell lines. Rabbit polyclonal antibodies against human AltPrP were raised against residues 59–73 and affinity purified by GenScript (Piscataway, NJ, USA). Secondary antibodies used were horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (NA931V) and HRP-conjugated donkey anti-rabbit IgG (NA934V; GE Healthcare, Little Chalfont, UK) and Alexa Fluor 488-conjugated goat anti-mouse IgG (A-11701) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (A-21069; Invitrogen). All other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

Cell culture and drug treatments

Human epithelial kidney (HEK293), murine neuroblastoma (N2a), and human astrocytoma (U-118 and U-87) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC, Canada). Human neuroblastoma [BE(2)-M17] cells were grown in a 1:1 mixture of Eagle's minimum essential medium and F-12 medium supplemented with 5% FBS. All culture media were supplemented with amphotericin B, as well as penicillin/streptomycin. Human primary neurons (1520-5; ScienCell, Carlsbad, CA, USA) were grown in neuronal medium (1521; ScienCell), according to the manufacturer's protocol. Peripheral blood mononuclear cells were purified from human peripheral blood using Ficoll-Paque PLUS (GE Healthcare), according to the manufacturer's protocol. Drug treatments were conducted on cells transfected for 24 h as follows, unless otherwise stated: MG132 and epoxomicin were used for 8 h, each at a concentration of 10 μ M. Thapsigargin, tunicamycin, and A23187 were used for 24 h at concentrations of 3.5, 1.5, and 1.3 μ M, respectively. Cycloheximide was used at a concentration of 107 μ M.

siRNA treatments

U-118 cells were plated in a 6-well plate at 2×10^5 cells/well in fresh medium containing no antibiotics. After 24 h, PRNP

siRNA (SI03019324) or AllStars Negative Control siRNA (1027281; Qiagen, Mississauga, ON, Canada) were transfected into the cells at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. After 72 h, cells were harvested, and lysates were processed for SDS-PAGE and Western blot analysis to assess knockdown efficiency.

Sample preparation and immunoblotting

Cells were grown in 6-well plates for 24 h and were then transfected as described above. Cells were rinsed and harvested in PBS and centrifuged for 60 s at 5000 rpm. Cells were then lysed in RIPA buffer, and samples were quantified using BCA protein assay reagent (Pierce, Waltham, MA, USA). Preparation of 10% human brain homogenate in PBS supplemented with EDTA Complete protease inhibitor cocktail (Roche Applied Science, Laval, QC, Canada) was prepared using an Omni TH115 tissue homogenizer (Omni International, Kennesaw, GA, USA), according to the manufacturer's protocol. Protein (100 μ g) from each sample was precipitated using the chloroform/methanol technique described by Wessel and Flugge (16), and the resulting pellets were resuspended in 4 \times SDS-PAGE loading dye. After electrophoresis, proteins were transferred to PVDF membranes, according to the manufacturer's protocol. Membranes were then exposed using Luminata Forte Western HRP Substrate (Millipore Corp., Billerica, MA, USA) or Western Lightning ECL reagent (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions. Films used were Amersham Hyperfilm ECL films (GE Healthcare). Membranes were stripped by washing twice in 0.2 N NaOH for 20 min, rinsed in PBS, blocked, and reprobed, as described above. Densitometric analysis was conducted using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Densitometric values were corrected for loading (anti-tubulin or anti-actin signal obtained by Western blot analysis).

Immunofluorescence

Immunofluorescence was carried out as described previously (17). Confocal analysis was carried out as described previously (18). Briefly, cells were examined with a scanning confocal microscope (FV1000; Olympus, Tokyo, Japan) coupled to an inverted microscope with a $\times 63$ oil-immersion objective (Olympus). Specimens were laser excited at 488 nm (40-mW argon laser) and 543 nm (diode laser). In order to avoid crosstalk, the emitted Alexa Fluor 488 and Alexa Fluor 568 fluorescences were collected sequentially at wavelengths 500–530 and >560 nm, respectively. Serial horizontal optical sections of 512×512 pixels with 2 times line averaging were taken at 0.11- μ m intervals through the entire thickness of the cell (optical resolution: lateral -0.18μ m; axial -0.25μ m). Images were acquired during the same day, using identical settings of the instrument. For illustration purposes, images were pseudocolored according to their original fluorochromes, merged (FluoView software; Olympus), then cropped and assembled (Adobe Photoshop software, Adobe Systems, Mountain View, CA, USA).

Mitochondrial fractionation

Mitochondria were isolated and treated with sodium carbonate or digitonin, as described previously (19). Briefly,

mitochondria were subjected to centrifugation at 13,000 g at 4°C for 10 min in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany). Mitochondria extracted with sodium

carbonate were pelleted by centrifugation at 600,000 g for 20 min at 4°C with a Beckman Coulter Optimax LX ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).

A

Human	AT	Ggc	ga-	---	--a	cct	tgg	ctg	ctg	gat	gct	ggt	tct	ctt	tgt	ggc	cac	atg	gag	tga	cct	ggg	cct	ctg	caa	
Chimpanzee	AT	Ggc	aa-	---	--a	cct	tgg	ctg	ctg	gat	gct	agt	tct	ctt	tgt	ggc	cac	atg	gag	tga	cct	ggg	cct	ctg	caa	
Macaque	AT	Ggc	ga-	---	--a	cct	tgg	ctg	ctg	gat	gct	ggt	tct	ctt	tgt	ggc	cac	atg	gag	tga	cct	ggg	cct	ctg	caa	
Cattle	AT	Ggt	gaa	aag	cca	cat	agg	cag	ttg	gat	cct	ggt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Bison	AT	Ggt	gaa	aag	cca	cat	agg	cag	ttg	gat	cct	ggt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Deer	AT	Ggt	gaa	aag	cca	cat	agg	cag	ctg	gat	cct	agt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Elk	AT	Ggt	gaa	aag	cca	cat	agg	cag	ctg	gat	cct	agt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Moose	AT	Ggt	gaa	aag	cca	cat	agg	cag	ctg	gat	cct	agt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Sheep	AT	Ggt	gaa	aag	cca	cat	agg	cag	ttg	gat	cct	ggt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Goat	AT	Ggt	gaa	aag	cca	cat	agg	cag	ttg	gat	cct	ggt	tct	ctt	tgt	ggc	cat	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Cat	AT	Ggt	gaa	agg	cca	cat	agg	cgg	ctg	gat	cct	ggt	tct	gtt	tgt	ggc	cac	atg	gag	tga	cgt	ggg	cct	ctg	caa	
Rabbit	AT	Ggc	g--	---	-ca	cct	cgg	cta	ctg	gat	gct	gct	tct	ctt	tgt	ggc	cac	gtg	gag	tga	cgt	ggg	cct	ctg	caa	
Mink	AT	Ggt	gaa	aag	cca	cat	agg	cag	ctg	gct	cct	ggt	tct	ctt	tgt	ggc	cac	atg	gag	tga	cat	tgg	ctt	ctg	caa	
Rat	AT	Ggc	ga-	---	--a	cct	tgg	cta	ctg	gct	gct	ggc	cct	ctt	tgt	gac	tac	atg	tac	tga	tgt	tgg	cct	ctg	caa	
Hamster	AT	Ggc	ga-	---	--a	cct	tag	ctt	ctg	gct	gct	ggc	act	ctt	tgt	ggc	tac	gtg	gac	tga	tgt	tgg	cct	ctg	caa	
Mouse	AT	Ggc	ga-	---	--a	cct	tgg	cta	ctg	gct	gct	ggc	cct	ctt	tgt	gac	tac	gtg	gac	tga	tgt	cgg	cct	ctg	caa	
Chicken	AT	Ggc	tag	gct	cct	cac	cac	ctg	ctg	cct	gct	ggc	cct	gct	gct	cgc	cgc	ctg	cac	cga	cgt	cgc	cct	ctc	caa	
Dog	AT	Ggt	gaa	aag	cca	cat	agg	cgg	ctg	gat	cct	gct	tct	ctt	tgt	ggc	cac	atg	gag	tga	cgt	ggg	cct	ctg	caa	
Pig	AT	Ggt	gaa	aag	cca	cat	agg	tgg	ctg	gat	cct	cgt	tct	ctt	tgt	ggc	cgc	atg	gag	tga	cat	agg	gct	ctg	caa	
Human		gaa	gcg	ccc	gaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	tgg	ggg	---	cag	ccg	ata	ccc	ggg	gca	ggg	cag	ccc	tgg	agg
Chimpanzee		gaa	gcg	ccc	gaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	tgg	ggg	---	cag	ccg	ata	ccc	ggg	gca	ggg	cag	ccc	tgg	agg
Macaque		gaa	gcg	ccc	aaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	tgg	agg	---	cag	ccg	ata	ccc	ggg	gca	ggg	cag	ccc	tgg	agg
Cattle		gaa	gcg	acc	aaa	acc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	agg	aca	ggg	cag	tcc	tgg	agg
Bison		gaa	gcg	acc	aaa	acc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	agg	aca	ggg	cag	tcc	tgg	agg
Deer		gaa	gcg	acc	aaa	acc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	ggg	aca	ggg	aag	tcc	tgg	agg
Elk		gaa	gcg	acc	aaa	acc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	ggg	aca	ggg	aag	tcc	tgg	agg
Moose		gaa	gcg	acc	aaa	acc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	ggg	aca	ggg	aag	tcc	tgg	agg
Sheep		gaa	gcg	acc	aaa	acc	tgg	cgg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	ggg	aca	ggg	cag	tcc	tgg	agg
Goat		gaa	gcg	acc	aaa	acc	tgg	cgg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	agg	aca	ggg	cag	tcc	tgg	agg
Cat		gaa	gcg	gcc	gaa	gcc	tgg	tgg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	gta	ccc	ggg	gca	ggg	cag	tcc	tgg	agg
Rabbit		gaa	gcg	gcc	gaa	gcc	tgg	agg	Agg	ATG	Gaa	cac	agg	ggg	---	gag	ccg	gta	ccc	ggg	tca	gag	cag	ccc	tgg	agg
Mink		gaa	gtg	gcc	aaa	gcc	tgg	agg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	agg	gca	ggg	cag	tcc	tgg	agg
Rat		aaa	gcg	gcc	aaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	tgg	tgg	---	aag	ccg	gta	ccc	tgg	gca	ggg	aag	ccc	tgg	agg
Hamster		gaa	gcg	gcc	aaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	tgg	cgg	---	aag	ccg	ata	ccc	tgg	gca	ggg	cag	ccc	tgg	agg
Mouse		aaa	gcg	gcc	aaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	cgg	tgg	---	aag	ccg	gta	tcc	cgg	gca	ggg	aag	ccc	tgg	agg
Chicken		gaa	ggg	c--	---	---	---	-aa	Agg	CAA	Acc	cag	tgg	tgg	---	ggg	ttg	ggg	cgc	cgg	gag	cca	tcg	cca	gcc	cag
Dog		gaa	gcg	gcc	gaa	gcc	t--	-gg	Agg	ATG	Gaa	cac	agg	ggg	tgg	gag	ccg	ata	ccc	agg	cca	ggg	TAG			
Pig		gaa	gcg	acc	aaa	gcc	tgg	cgg	Agg	ATG	Gaa	cac	tgg	ggg	---	gag	ccg	ata	ccc	agg	gca	ggg	TAG			
Human		caa	ccg	cta	ccc	acc	tca	ggg	cgg	tgg	tgg	ctg	ggg	gca	gcc	tca	t--	-gg	tgg	tgg	ctg	---	---	---	---	
Chimpanzee		caa	ccg	cta	ccc	acc	tca	ggg	cgg	tgg	tgg	ctg	ggg	gca	gcc	tca	t--	-gg	tgg	tgg	ctg	---	---	---	---	
Macaque		caa	ccg	cta	ccc	acc	tca	ggg	tgg	tgg	tgg	ctg	ggg	gca	gcc	tca	t--	-gg	tgg	tgg	ctg	---	---	---	---	
Cattle		caa	ccg	tta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	ggg	cca	gcc	tca	tgg
Bison		caa	ccg	tta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	ggg	cca	gcc	tca	tgg
Deer		caa	ccg	cta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	---	---	---	---	
Elk		caa	ccg	cta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	---	---	---	---	
Moose		caa	ccg	cta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	---	---	---	---	
Sheep		caa	ccg	cta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	---	---	---	---	
Goat		caa	ccg	cta	tcc	acc	tca	ggg	agg	ggg	tgg	ctg	ggg	tca	gcc	cca	t--	-gg	agg	tgg	ctg	---	---	---	---	
Cat		caa	ccg	tta	ccc	acc	cca	ggg	cgg	tgg	cgg	ctg	ggg	tca	gcc	cca	cgc	cgg	cgg	cgg	ctg	---	---	---	---	
Rabbit		caa	ccg	cta	ccc	acc	cca	ggg	cgg	tgg	cgg	ctg	ggg	aca	gcc	cca	t--	-gg	cgg	cgg	cgg	ctg	---	---	---	---
Mink		caa	ccg	cta	ccc	acc	cca	ggg	tgg	tgg	cgg	ctg	ggg	cca	gcc	cca	c--	-gg	ggg	tgg	ctg	---	---	---	---	
Rat		caa	ccg	tta	ccc	acc	tca	gag	tgg	tgg	tac	ctg	ggg	gca	gcc	cca	t--	-gg	tgg	tgg	ctg	---	---	---	---	
Hamster		caa	ccg	tta	ccc	acc	tca	ggg	---	---	---	---	---	---	---	---	t--	-gg	cgg	cac	ctg	---	---	---	---	
Mouse		caa	ccg	tta	ccc	acc	tca	ggg	---	---	---	---	---	---	---	---	---	---	tgg	cac	ctg	---	---	---	---	
Chicken		cta	ccc	ccg	cca	gcc	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
Dog																										
Pig																										
Human		---	---	---	ggg	gca	gcc	tca	tg-	--g	tgg	tgg	ctg	ggg	gca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	tca
Chimpanzee		---	---	---	ggg	gca	gcc	tca	tg-	--g	tgg	tgg	ctg	ggg	gca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	tca
Macaque		---	---	---	ggg	gca	acc	cca	tg-	--g	tgg	cgg	ctg	ggg	aca	gcc	tca	tg-	--g	tgg	cgg	ctg	ggg	aca	gcc	tca
Cattle		agg	tgg	ctg	ggg	cca	gcc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	aca
Bison		agg	tgg	ctg	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	aca
Deer		---	---	---	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	gca	gcc	aca
Elk		---	---	---	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	aca

Figure 1. An alternative ORF overlapping with the PrP octarepeat (OR) region exists in the +3 frame of several species. A) DNA sequence alignment of *Prnp* in several species. *Prnp* DNA sequences of several species were aligned starting from the PrP start codon (capitalized letters) until the AltPrP stop codon (boxed and capitalized). All larger mammals analyzed contain a start codon (boxed and capitalized) surrounded by a Kozak consensus sequence (underscored). Most smaller species analyzed do not possess a conventional ATG start codon (shown in gray), though they all possess a Kozak consensus sequence, except chicken. Sequences were obtained from GenBank and aligned using ClustalW software. GenBank accession number of each sequence (continued on next page)

After digitonin treatment, mitoplasts were pelleted by centrifugation at 13,000 *g* at 4°C for 10 min in an Eppendorf centrifuge.

PNGase F treatment and solubility assay

PNGase F treatment of transfected cell lysates (New England Biolabs) was carried out according to the manufacturer's instructions. Briefly, 100 µg of proteins extracted in RIPA buffer was adjusted to 0.5% SDS, boiled 10 min, and digested with 2 U of PNGase F for 18 h at 37°C. Solubility of PrP was assessed as described previously (20).

Ethics statement

The human brain sample was a kind gift from Dr. Cheryl Wellington (Department of Pathology, University of British Columbia, Vancouver, BC, Canada). All experiments were performed according to University of British Columbia ethics protocol UBC C04-0595.

Bioinformatics

DNA sequences of *Prnp* in several species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) and aligned using ClustalW software (<http://www.clustal.org>).

Moose	---	---	---	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	gca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	gca	gcc	aca
Sheep	---	---	---	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	aca
Goat	---	---	---	ggg	cca	acc	tca	tg-	--g	agg	tgg	ctg	ggg	tca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	aca
Cat	---	---	---	ggg	tca	gcc	cca	cgc	cgg	cgg	agg	ctg	ggg	tca	gcc	cca	cgc	cgg	cgg	tgg	ctg	ggg	tca	gcc	cca
Rabbit	---	---	---	ggg	gca	gcc	tca	cg-	--g	tgg	tgg	ctg	ggg	aca	gcc	cca	cg-	--g	cgg	tgg	ctg	ggg	gca	gcc	cca
Mink	---	---	---	ggg	aca	gcc	cca	cg-	--g	ggg	tgg	ctg	ggg	tca	gcc	cca	cg-	--g	ggg	tgg	ctg	ggg	aca	gcc	gca
Rat	---	---	---	ggg	aca	acc	tca	tg-	--g	tgg	tgg	ctg	ggg	aca	acc	tca	tg-	--g	tgg	tgg	ctg	ggg	tca	gcc	cca
Hamster	---	---	---	ggg	gca	acc	cca	tg-	--g	tgg	tgg	ctg	ggg	aca	gcc	cca	tg-	--g	tgg	tgg	ctg	ggg	tca	gcc	cca
Mouse	---	---	---	ggg	gca	gcc	cca	cg-	--g	tgg	tgg	ctg	ggg	aca	acc	cca	tg-	--g	ggg	cag	ctg	ggg	aca	acc	tca
Chicken	---	---	---	ggg	cta	ccc	tca	TAA																	
Dog																									
Pig																									

Human	t--	-gg	tgg	tgg	ctg	ggg	tca	agg	agg	tgg	cac	cca	cag	tca	gtg	gaa	caa	gcc	gag	TAA				M13899
Chimpanzee	t--	-gg	tgg	tgg	ctg	ggg	tca	agg	agg	tgg	cac	cca	cag	tca	gtg	gaa	caa	gcc	gag	TAA				AY665268
Macaque	t--	-gg	tgg	tgg	ctg	ggg	tca	agg	agg	tgg	cac	cca	caa	tca	gtg	gca	caa	gcc	cag	TAA				U08307
Cattle	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	t--	-ac	cca	cgg	tca	atg	gaa	caa	acc	cag	TAA				AB001468
Bison	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	t--	-ac	cca	cgg	tca	atg	gaa	caa	acc	cag	TAA				AY769958
Deer	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	t--	-ac	cca	cag	tca	gtg	gaa	caa	gcc	cag	TAA				AY286008
Elk	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	t--	-ac	cca	cag	tca	gtg	gaa	caa	gcc	cag	TAA				AF016228
Moose	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	t--	-ac	cca	cag	tca	gtg	gaa	caa	gcc	cag	TAA				AY225484
Sheep	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	TAG														U67922
Goat	tgg	tgg	tgg	agg	ctg	ggg	tca	agg	tgg	TAG														S82626
Cat	cgc	cgc	cgc	cgc	ctg	ggg	tca	agg	tgg	tgg	cac	cca	cgg	tca	gtg	ggg	caa	acc	cag	TAA				AF003087
Rabbit	c--	-gg	tgg	tgg	ctg	ggg	tca	agg	agg	tgg	tac	cca	caa	cca	gtg	ggg	caa	gcc	cag	TAA				AF015603
Mink	tgg	tgg	cgg	tgg	ctg	ggg	tca	agg	tgg	tgg	gag	cca	cgg	tca	gtg	ggg	caa	gcc	cag	TAA				EF508270
Rat	t--	-gg	cgg	ggg	ctg	gag	tca	agg	agg	ggg	tac	cca	TAA											D50093
Hamster	t--	-gg	tgg	tgg	ctg	ggg	tca	agg	agg	tgg	cac	cca	caa	tca	gtg	gaa	caa	gcc	car	TAA				M14054
Mouse	t--	-gg	tgg	TAG																				M18071
Chicken																								GU991271
Dog																								AF042843
Pig																								AY835633

B

Human	MEHWG-QPIPGAGQPWRLPLSTSGRWLGAAS-WWWLGA-----ASWWWLGAAP-WWWLGTASWW-W-LGSRWWHPQSVEQAE	73
Chimpanzee	MEHWG-QPIPGAGQPWRLPLSTSGRWLGAAS-WWWLGA-----ASWWWLGAAP-WWWLGTASWW-W-LGSRWWHPQSVEQAE	73
Macaque	MEHWR-QPIPGAGQPWRLPLTPGWLGAAS-WWWLGA-----TPWWRLGTAS-WWRLGTASWW-W-LGSRWWHPQSVAQAE	73
Cattle	MEHWG-EPIPTGQSWRQPLSTSGRWLGSAP-WRWLGPASWRWLGPSWRWLGSA-WWWLGTATWW-WRLGSR-WYPRSMETQ	81
Bison	MEHWG-EPIPTGQSWRQPLSTSGRWLGSAP-WRWLGPASWRWLGPTSWRWLGSAP-WWWLGTATWW-WRLGSR-WYPRSMETQ	81
Deer	MEHWG-EPIPTGKSWRQPLSTSGRWLGSAP-WRWLGP-----TSWRWLGSAP-WWWLGAATWW-WRLGSR-WYPQSVEQAE	73
Elk	MEHWG-EPIPTGKSWRQPLSTSGRWLGSAP-WRWLGP-----TSWRWLGSAP-WWWLGTATWW-WRLGSR-WYPQSVEQAE	73
Moose	MEHWG-EPIPTGKSWRQPLSTSGRWLGSAP-WRWLGP-----TSWRWLGAAP-WWWLGAATWW-WRLGSR-WYPQSVEQAE	73
Sheep	MEHWG-EPIPTGQSWRQPLSTSGRWLGSAP-WRWLGP-----TSWRWLGSAP-WWWLGTATWW-WRLGSR-W	64
Goat	MEHWG-EPIPTGQSWRQPLSTSGRWLGSAP-WRWLGP-----TSWRWLGSAP-WWWLGTATWW-WRLGSR-W	64
Cat	MEHWG-EPVPGQSWRQPLPTPGRWRLGSAPRRRLGS-A-----PRRRRLGSAPRRRLGSAPRR-RRLGSRWWHPRSVGQTQ	77
Rabbit	MEHRG-EPVPGSEQPWRLPLPTPGRWRLGTAP-WRRLGA-----ASRWLGTAP-RRWLGAAPRW-W-LGSRWWYPQVPGQAE	73
Mink	LEHWG-EPIPRAGQSWRQPLPTPGWRLGPAP-RGWLGT-----APRGWLGSAP-RGWLGTAAWRRW-LGSRWWEPRSVGQAE	74
Rat	VEHWW-KPVPWAGKPWRQPLPTSEWYWLGAAP-WWWLGT-----TSWWWLGTTS-WWWLGSAPRW-G-LESRRGYP	66
Hamster	VEHWR-KPIPWAGQPWRLPLPTSG-----WRHLGA-----TPWWLGTAP-WWWLGSAPWW-W-LGSRWWHPQSVEQAX	65
Mouse	VEHWR-KPVSRAKFPWRQPLPTSG-----WHLGA-----APRWLGTTP-WQGLGTTSWW	48
Chicken	-----RLHRRRPLQEQGRQ-----TQWWGLGRRE-----PSPAQL-----PPPAGLPS	38
Dog	MEHRGWEPIPRG	13
Pig	MEHWG-EPIPRAG	12

Figure 1. (continued) used is included at the end of the DNA sequence. *B*) Amino acid sequence alignment of AltPrP in several species. AltPrP is present in several large mammals and has conserved tryptophan-rich repeats. Some smaller mammals contain the AltPrP ORF, yet have interrupted tryptophan-rich repeats replaced by arginine (such as cat and rabbit). Other species do not possess a conventional initiator methionine residue (mink, rat, hamster, and mouse) or contain a stop codon early on in the peptide sequence (dog and pig). Length of the ORF in each species is indicated at the end of the peptide sequence. Sequences were translated from DNA sequences obtained from GenBank using the ExPaSy Translate tool and then aligned using ClustalW software. Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under accession numbers TPA: BK007887–BK007890.

TABLE 2. Amino acid sequences of constructs used to detect AltPrP

Construct	Reading frame	Sequence
huPrP	1	MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPHGGG- WGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGY- MLGSAMSRPIIHFGSDYEDRYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTVTTT- TKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSPPVILLISFLIFLIVG
	3	MEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRRW- HPQSVEQAE
huPrP ^(HA)	1	MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPHGG- GWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHSQWNKPS <u>IRTTYQTTPKPKTNMKHMAGAAA-</u> AGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYRENMHRYPNQVYYRPMDEYSNQNNFVHDCV- NITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSPPVILL- ISFLIFLIVG
	3	MEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRR- WHPQSVEQAEYPYDVPDYA
huPrP ^{(HA)*}	1	MANLGCWMLVLFVATWSDLGLCKKRPKPGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPHGG- GWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHSQWNKPS <u>IRTTYQTTPKPKTNMKHMAGAAA-</u> AGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYRENMHRYPNQVYYRPMDEYSNQNNFVHDCV- NITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSPPVILL- ISFLIFLIVG
	3	<u>LEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRRW-</u> HPQSVEQAE
huAltPrP	1	MEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRRW- HPQSVEQAE
huAltPrP ^{HA}	1	MEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRRW- HPQSVEQAEYPYDVPDYA
PrP ^{Δ1–66} (HA)	1	MGLCKKRPKPGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPHGGGGWGQPHGGGGWGQPHGGG- GQPHGGGGWGQGGGTHSQWNKPS <u>IRTTYQTTPKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAM-</u> RPIIHFGSDYEDRYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIKQHTVTTTTKGENF- TETDVKMMERVVEQMCITQYERESQAYYQRGSSMVLFSPPVILLISFLIFLIVG
	3	MEHWGQPIPGAGQPWRQPLPTSGRWLGTASWWLGAASWWLGAAPWWLGTASWWLGSRRW- HPQSVEQAEYPYDVPDYA
boPrP	1	MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPH- GGGWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHGQWNKPSKPKTNMKHVAGAAA- AGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYRENMHRYPNQVYYRPVDQYSNQNNFVHDCVN- ITVKEHTVTTTTKGENFTETDIKMMKRVEQMCITQYQRESQAYYQRGASVILFSPPVILLIS- FLIFLIVG
	3	MEHWGEPIPTGQSWRQPLSTSGRGWLGSAPWRWLGPAWRWLGPAWRWLGSAWVWLGTATW- WWRLGSRWYPRSMETQ
boPrP ^(HA)	1	MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPH- GGGWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHGQWNKPS <u>IRTTYQTTLKPKTN-</u> MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYRENMHRYPNQVYYRPVDQYSNQ- NNFVHDCVNITVKEHTVTTTTKGENFTETDIKMMKRVEQMCITQYQRESQAYYQRGASVILFS- SPPVILLISFLIFLIVG
	3	MEHWGEPIPTGQSWRQPLSTSGRGWLGSAPWRWLGPAWRWLGPAWRWLGSAWVWLGTATW- WWRLGSRWYPRSMETQYPYDVPDYA
boAltPrP ^{HA}	1	MEHWGEPIPTGQSWRQPLSTSGRGWLGSAPWRWLGPAWRWLGPAWRWLGSAWVWLGTATW- WWRLGSRWYPRSMETQYPYDVPDYA
wtPrP	1	MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPH- GGGWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHSQWNKPSKPKTNMKHVAGAAAAAGAVVG- GGYMLGSAMSRPLIHFGNDYEDRYRENMYRPNQVYYRPVDQYNNQNTFVHDCVNITVKQHTV- TTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQRGASVILESSPPVILLISFLIFLIVG
	3	MEHWGEPIPTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAWVWLGAATWWRLGSRW- YPQSVEQAQ
wtPrP ^(HA)	1	MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPPGNNRYPPQGGGGWGQPH- GGGWGQPHGGGGWGQPHGGGGWGQPHGGGGWGQGGGTHSQWNKPS <u>IRTTYQTTLKPKTNMKHVAGAA-</u> AAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYRENMYRPNQVYYRPVDQYNNQNTFVHDCV- NITVKQHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQRGASVILESSPPVILLI- SFLIFLIVG
	3	MEHWGEPIPTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAWVWLGAATWWRLGSRW- YPQSVEQAQYPYDVPDYA
wtAltPrP ^{HA}	1	MEHWGEPIPTGKSWRQPLSTSGRGWLGSAPWRWLGPTSWRWLGSAWVWLGAATWWRLGSRW- YPQSVEQAQYPYDVPDYA

(continued on next page)

TABLE 2. (continued)

Construct	Reading frame	Sequence
shPrP		MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQSGPQGNRYPPQGGGGWGQPH -
	1	GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGSHSQWNKPSKPKTNMKHVAGAAAAAGAVVGGL -
	3	GGYMLGSAMSRPLIHFGNDYEDRYRENMYRYPNQVYYRPVDQYSNQNNFVHDCVNIIVKQHTV -
shPrP ^(HA)	1	TTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLISFLIFLIVG
		MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPSTSWRWLGSAPWWLGTATWWRLGSRW
	3	MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQSGPQGNRYPPQGGGGWGQPH -
shAltPrP ^{HA}	1	GGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG <i>IRTTYQTTP</i> SHSQWNKPSKPKTNMKHVAGAA -
		AAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYRENMYRYPNQVYYRPVDQYSNQNNFVHDCV -
	3	NITVKQHTVTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQRGASVILFSSPPVILLI -
		SFLIFLIVG
		MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPSTSWRWLGSAPWWLGTATWWRLGSR -
		<u>WYPYDVPDYA</u>
	1	MEHWGEPIPGTGQSWRQPLSTSGRGWLGSAPWRWLGPSTSWRWLGSAPWWLGTATWWRLGSR -
		<u>WYPYDVPDYA</u>

Amino acid sequences of all constructs used are included. Note that wherever possible, the amino acid sequence of both the PrP reading frame (+1) and the AltPrP reading frame (+3) are included for each construct. Underscored letters represent the hemagglutinin (HA) tag. When shown in roman type, the HA sequence is in its proper reading frame in order to be detected by an anti-HA antibody. When shown in italics, the HA sequence is in another reading frame, which cannot be detected by an anti-HA antibody. Note that for huPrP^{(HA)*}, the initiator methionine codon (M) is replaced by leucine (L, double underscored).

DNA sequences were translated using the ExPaSy Translate tool (<http://expasy.org>).

RESULTS

An alternative ORF exists in the coding sequence of PrP

In an attempt to uncover the reason for the numerous functions attributed to PrP, we have reexamined the sequence of *PRNP* from several species. We noticed an ORF in the +3 reading frame, in which the AUG initiation codon (bp 90–92 in the human PrP CDS) is positioned within an optimal Kozak context (Fig. 1A). We termed the putative polypeptide encoded by this ORF alternative PrP (AltPrP). The AltPrP ORF covers the entire octarepeat (OR) region of PrP. AltPrP ranges in length between 64 and 81 aa, and contains several tryptophan-rich repeats resulting from translation of the OR region of PrP in the +3 reading frame (Fig. 1B). In other species, such as mouse and hamster, the alternative initiation AUG codon is absent and is replaced by a GUG codon still located within an optimal Kozak sequence (Fig. 1A). Although there is at least one example in which GUG is an efficient initiation codon to translate a protein (21), we focused on the expression of AltPrP in humans, cattle, sheep, and white-tailed deer in this study.

AltPrP is expressed from PrP cDNA of several species

A detailed list of all constructs used, along with amino acid sequences of each construct, is outlined in Table 2. To test whether AltPrP is expressed, we introduced a hemagglutinin (HA) tag in frame with AltPrP to pro-

duce carboxy-tagged AltPrP (AltPrP^{HA}), within the human PrP cDNA (Fig. 2A). For clarity reasons, this construct is termed PrP^(HA), where (HA) indicates that the HA tag is silent within the reading frame of PrP. As a positive control, we created a construct encoding solely AltPrP^{HA}. We also engineered a PrP^{(HA)*} construct with an inactivated alternative initiation codon. In this construct, termed PrP^{(HA)*}, the AUG at bp 90 was changed to CUG. Lysates from mammalian cells transfected with PrP, PrP^(HA), or PrP^{(HA)*} were probed with both anti-PrP and anti-HA antibodies to test for the expression of PrP, PrP^(HA), PrP^{(HA)*}, and AltPrP^{HA}. As expected, the introduction of the HA tag resulted in a slight increase in the molecular weight of PrP (Fig. 2B). On a SDS-PAGE gel, PrP^(HA) migrated as several bands, indicating the presence of glycosylations similar to native PrP. Remarkably, a band corresponding to the expected molecular weight for AltPrP^{HA} was detected with an anti-HA antibody in cells transfected with PrP^(HA). The identity of this band was confirmed by probing lysates from cells directly transfected with cDNA encoding AltPrP^{HA}. AltPrP^{HA} was not detected in cells transfected with PrP^{(HA)*} (Fig. 2B), clearly showing that translation of AltPrP is indeed initiated at the identified alternative AUG codon (Fig. 1A), most likely by alternative translation initiation. Analogous results were obtained in neuronal, as well as in non-neuronal cell lines (Fig. 2B). We then confirmed these results by immunofluorescence on N2a and HEK293 cells expressing each of these constructs (Fig. 2C). Detection of PrP, PrP^(HA), PrP^{(HA)*}, and AltPrP^{HA} was done using anti-PrP and anti-HA antibodies. In both cell types, expression of PrP^(HA) and AltPrP^{HA} resulted in an HA signal with a granular cytoplasmic distribution. Expression of PrP or PrP^{(HA)*} did not provide an HA signal, confirming the results described in Fig. 2B. The addition of the out-of-frame HA tag in PrP^(HA) and PrP^{(HA)*} did not affect the subcellular localization of PrP, which

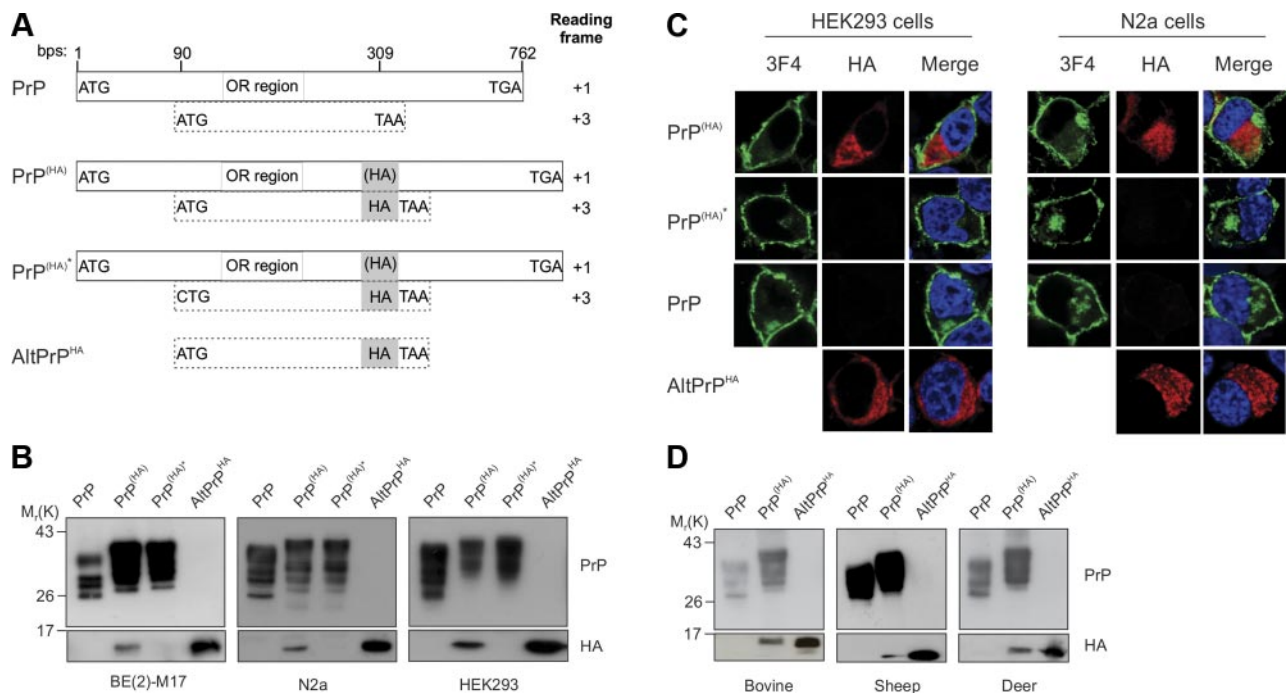


Figure 2. Detection of AltPrP. **A**) Strategy used to detect AltPrP by introducing an HA tag at the C-terminus of AltPrP. AltPrP is in the +3 reading frame in relation to PrP and spans the entire OR region in the N-terminal domain of PrP. Top box in each construct represents the PrP reading frame (+1); dashed bottom box represents the AltPrP reading frame. In PrP^(HA), PrP^{(HA)*}, and AltPrP^{HA}, an HA tag (shaded box) was inserted at the C terminus of AltPrP. Parentheses surrounding the HA in the PrP reading frame represent the fact that the HA epitope sequence is encoded in the AltPrP reading frame, and is, therefore, undetected if expressed from the ATG codon at bp 1 of the PrP CDS. PrP^{(HA)*} is identical to PrP^(HA) except that the ATG codon at bp 90 has been mutated to CUG. **B**) Western blot against PrP (3F4 epitope) and AltPrP (HA epitope) in BE(2)-M17, N2a, and HEK293 cells transfected with PrP, PrP^(HA), PrP^{(HA)*}, and AltPrP^{HA} constructs. **C**) Cells transfected with PrP, PrP^(HA), PrP^{(HA)*}, or AltPrP^{HA} were immunostained with anti-PrP (3F4; green) or anti-HA (red) antibodies. Merged images also show nuclei stained with Hoechst (blue). **D**) Expression of AltPrP^{HA} from bovine, sheep, and deer cDNA PrP constructs. Note the slight change in molecular weight between species.

is typically localized at the plasma membrane and the Golgi apparatus.

The ORF encoding AltPrP is also present in *PRNP* from cattle, sheep, and white-tailed deer (Fig. 1A). An HA tag was inserted at the C terminus of AltPrP in PrP cDNA of each of these species using the same strategy as above for human PrP. Western blot analysis of HEK293 cells transfected with cattle, sheep, and deer PrP^(HA) cDNA demonstrated the presence of AltPrP in these 3 species, in addition to humans (Fig. 2D). These data clearly demonstrate that under normal conditions, the prion protein cDNA of numerous species directs the synthesis of 2 coexpressed polypeptides: PrP and the newly identified AltPrP.

AltPrP is a mitochondrial protein

We then addressed the subcellular localization of AltPrP by immunofluorescence. The precise cytoplasmic localization of AltPrP was determined in both N2a and HEK293 cells expressing human AltPrP^{HA} using antibodies against different cytoplasmic organelles. We observed a clear colocalization of AltPrP^{HA} with mitochondrial Hsp70 (Fig. 3A) and cytochrome *c* (data not shown). The presence of AltPrP at the mitochondria was confirmed by subcellular fractionation and differ-

ential centrifugation of HEK293 cells stably transfected with AltPrP^{HA}. AltPrP^{HA} was detected in the crude cellular fraction, as well as in the mitochondrial fraction, but not in the cytosol (Fig. 3B). Bioinformatic analysis predicts a transmembrane domain within AltPrP (Fig. 3C). To test this hypothesis, mitochondria isolated from cells expressing AltPrP^{HA} were treated with sodium carbonate. Similar to other mitochondrial membrane-integrated proteins, including VDAC and Cox IV, AltPrP was not extracted after alkali treatment. In contrast, Bax, a peripheral mitochondrial membrane protein in nonapoptotic cells (19) was completely extracted by treatment with sodium carbonate (Fig. 3D). This result demonstrates that AltPrP is a mitochondrial membrane-integrated protein. We next tested whether AltPrP is inserted in the mitochondrial inner or outer membrane with digitonin to selectively solubilize the outer membrane (19). In the presence of digitonin, most of the VDAC (an outer membrane component) was extracted from mitochondria, while Cox IV (localized at the inner membrane) remained associated with the mitochondria. In these conditions that allow selective extraction of the outer membrane, AltPrP did not remain attached to the mitochondria (Fig. 3E), indicating that AltPrP is inserted in the outer mitochondrial membrane.

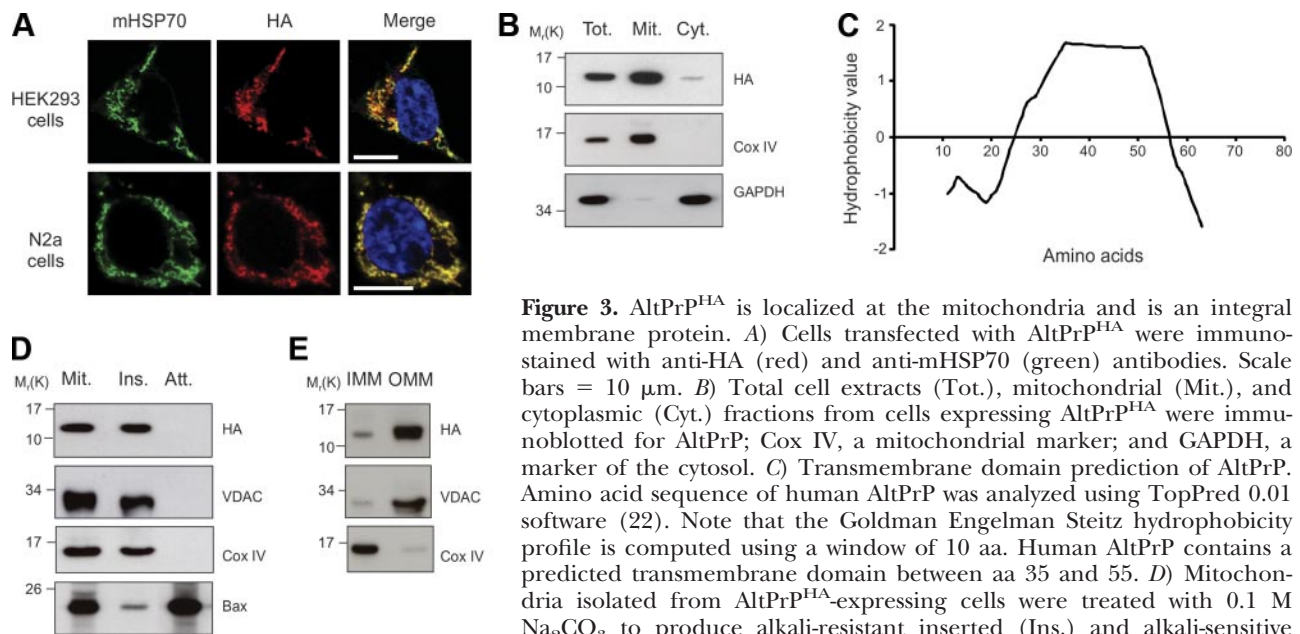


Figure 3. AltPrP^{HA} is localized at the mitochondria and is an integral membrane protein. *A*) Cells transfected with AltPrP^{HA} were immunostained with anti-HA (red) and anti-mHSP70 (green) antibodies. Scale bars = 10 μ m. *B*) Total cell extracts (Tot.), mitochondrial (Mit.), and cytoplasmic (Cyt.) fractions from cells expressing AltPrP^{HA} were immunoblotted for AltPrP; Cox IV, a mitochondrial marker; and GAPDH, a marker of the cytosol. *C*) Transmembrane domain prediction of AltPrP. Amino acid sequence of human AltPrP was analyzed using TopPred 0.01 software (22). Note that the Goldman Engelman Steitz hydrophobicity profile is computed using a window of 10 aa. Human AltPrP contains a predicted transmembrane domain between aa 35 and 55. *D*) Mitochondria isolated from AltPrP^{HA}-expressing cells were treated with 0.1 M Na₂CO₃ to produce alkali-resistant inserted (Ins.) and alkali-sensitive attached (Att.) fractions, and were analyzed by Western blot for AltPrP, VDAC, Cox IV, and Bax. *E*) After treatment with 0.2 mg/ml digitonin, the digitonin-sensitive outer mitochondrial membrane (OMM) and digitonin-resistant inner mitochondrial membrane (IMM) fractions were analyzed by Western blot for the presence of AltPrP^{HA}, VDAC, and Cox IV.

VDAC, Cox IV, and Bax. *E*) After treatment with 0.2 mg/ml digitonin, the digitonin-sensitive outer mitochondrial membrane (OMM) and digitonin-resistant inner mitochondrial membrane (IMM) fractions were analyzed by Western blot for the presence of AltPrP^{HA}, VDAC, and Cox IV.

AltPrP expression is increased by proteasome inhibition and endoplasmic reticulum (ER) stress

The observation that expression levels of AltPrP^{HA} in cells transfected with PrP^(HA) are lower than levels in cells directly transfected with AltPrP^{HA} suggests that expression of AltPrP from PrP cDNA is constitutively negatively regulated (Fig. 2). Certain cellular stresses modulate the expression level of a great number of proteins (23, 24). Because AltPrP originates from *PRNP*, a locus linked to neurodegeneration, we decided to test whether AltPrP expression levels were affected by stresses implicated in neurodegenerative diseases. One such example is proteasomal dysfunction, which is often associated with neurodegeneration (25, 26). Treatment of HEK293 cells expressing PrP^(HA) or AltPrP^{HA} with the proteasome inhibitors MG132 or epoxomicin for 8 h resulted in a 2- to 3-fold increase of AltPrP^{HA} levels (Fig. 4A, B). Neither drug modified the levels of PrP^(HA) or tubulin in the same experimental conditions, showing that this increase is specific to AltPrP. The effect of MG132 was rapid, since a significant increase of AltPrP^{HA} levels was observed as soon as 2 h after the addition of the drug (Fig. 4C), an indication that AltPrP is a naturally labile protein with a short half-life, probably degraded in a proteasome-dependent manner. The half-life of AltPrP was determined in the presence of cycloheximide, an inhibitor of protein synthesis. In the absence of MG132, the half-life of AltPrP was estimated to be <0.5 h (Fig. 4D). The half-life of AltPrP in the presence of MG132 was >2 h, confirming its stabilization by proteasome inhibition.

Another stress associated with neurodegenerative disorders is ER stress (27, 28). It is known that the rate

of alternative translation initiation in several mRNAs can be significantly increased during ER stress (29, 30). Since AltPrP is most likely produced by alternative translation initiation (Fig. 2B), we tested whether ER stress could modulate its expression. HEK293 cells expressing PrP^(HA) or AltPrP^{HA} were treated with drugs known to perturb ER homeostasis: thapsigargin (an ER-calcium ATPase inhibitor), tunicamycin (an N-glycosylation inhibitor), and A23187 (a calcium ionophore). The 3 drugs induced an ER stress, as monitored by interfered glycosylation of PrP (demonstrated by the disappearance of higher molecular-weight bands detected by the anti-PrP antibody), as well as increased levels of BiP/GRP78 chaperone (Fig. 5A; refs 31, 32). All 3 drugs also induced a ≥ 2 -fold increase in AltPrP^{HA} levels in cells expressing PrP^(HA). However, this effect was not observed in cells directly transfected with AltPrP^{HA}, in which AltPrP^{HA} is no longer produced by alternative translation initiation at a downstream AUG codon (Fig. 5A, E). We concluded that ER stress specifically increases the synthesis of AltPrP from PrP cDNA.

We hypothesized that the target of this regulation is located upstream of the AltPrP start codon (bp 1–90 of the human PrP CDS). If so, this region is expected to exert an inhibitory effect on the translation of AltPrP from PrP cDNA that might be abolished during ER stress. To test this hypothesis, we engineered a mutant cDNA construct, termed PrP ^{Δ 1–66(HA)}, with a deletion of the first 66 bp of the PrP CDS. Since bp 1–66 encode the N-terminal signal peptide, PrP ^{Δ 1–66(HA)} is expressed as a cytoplasmic protein and does not undergo post-translational modifications, migrating as a single band (Fig. 5B). AltPrP^{HA} was constitutively expressed in cells transfected with PrP ^{Δ 1–66(HA)} similarly to cells trans-

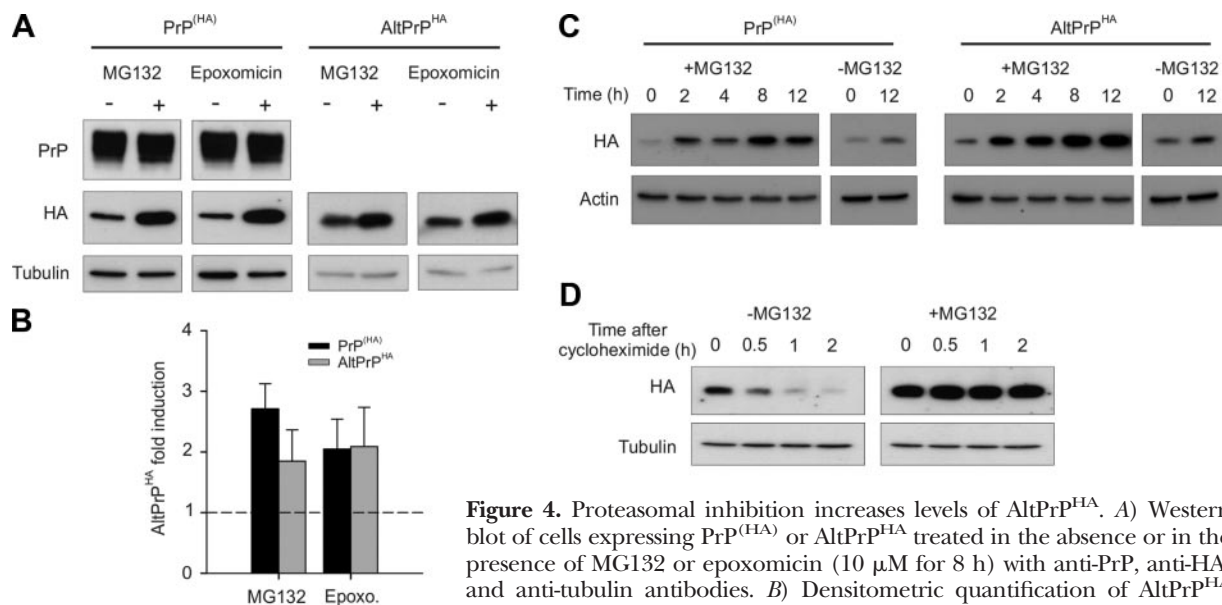


Figure 4. Proteasomal inhibition increases levels of AltPrP^{HA}. **A)** Western blot of cells expressing PrP^(HA) or AltPrP^{HA} treated in the absence or in the presence of MG132 or epoxomicin (10 μ M for 8 h) with anti-PrP, anti-HA, and anti-tubulin antibodies. **B)** Densitometric quantification of AltPrP^{HA} induction following proteasome inhibition. Note that both MG132 and epoxomicin induce 2- to 3-fold increase of AltPrP^{HA} whether expressed from PrP^(HA) or AltPrP^{HA}. No significant differences (*t* test) in induction of AltPrP^{HA} could be observed between the 2 transfection conditions, regardless of the proteasome inhibitor used. Dotted line represents AltPrP^{HA} levels in untreated transfected cells normalized to 1. Values are expressed as means \pm SD from ≥ 3 independent experiments. **C)** Time course analysis of MG132-mediated proteasome inhibition on the accumulation of AltPrP in cells expressing PrP^(HA) or AltPrP^{HA}. Cell extracts were immunoblotted with anti-HA and anti-actin antibodies. **D)** AltPrP^{HA}-expressing cells preincubated with MG132 (10 μ M for 6 h) were treated with cycloheximide for various times and immunoblotted for HA and tubulin.

fecting with PrP^(HA). More important, induction of AltPrP^{HA} by thapsigargin was abolished, confirming that bp 1–66 comprise at least part of the regulation domain for AltPrP expression from PrP cDNA (Fig. 5B, E).

Other stresses, including heat shock and oxidative stress, did not induce any change in the levels of AltPrP. In these experiments, cellular stress was monitored by increased levels of phospho-eIF2 α (Fig. 5C–E). Therefore, the synthesis of AltPrP from PrP cDNA is specifically regulated by ER stress.

Absence of AltPrP has no obvious effect on PrP biology

Because PrP and AltPrP are produced from the same gene, it was important to test for a direct functional relation between these proteins. We verified whether the absence of AltPrP would alter the biology of PrP, namely its post-translational modifications, localization, and solubility. Since AltPrP expression is abolished when the AUG codon at bp 90 is mutated (Fig. 2B), we compared cells expressing either PrP^{(HA)*} with cells expressing PrP^(HA) to answer these questions. As a control, we also included cells transfected with wild-type PrP to ensure that each treatment was performed properly. PNGase F is a specific N-glycosidase commonly used to deglycosylate PrP in order to characterize its post-translational modifications. After PNGase F treatment of lysates of HEK293 cells transfected with human PrP, PrP^(HA), and PrP^{(HA)*}, only the unglycosylated band was detected by Western blot (data not shown). Thus, neither the HA tag nor the coexpression of AltPrP interfered with post-translational modifica-

tions of PrP. Similarly, AltPrP did not perturb the trafficking of PrP, since both PrP^(HA) and PrP^{(HA)*} were mainly localized at the plasma membrane and in the Golgi apparatus (Fig. 2C). Finally, an essential characteristic of PrP is its ability to switch from a soluble into a disease-associated insoluble isoform (20). To test whether the solubility of PrP was affected by coexpression of AltPrP, we monitored PrP, PrP^(HA), and PrP^{(HA)*} solubility in HEK 293 cells by ultracentrifugation. Each construct displayed the same solubility (data not shown). We concluded that the presence of AltPrP has no apparent effect on the biology of PrP. This result was not unexpected, since under normal conditions, PrP and AltPrP are not localized in the same cellular compartments and are therefore unlikely to interact.

AltPrP is endogenously expressed from *PRNP*

Next, we raised and affinity-purified a polyclonal antibody against the C terminus of human AltPrP to detect wild-type AltPrP encoded by the endogenous *PRNP* gene. The anti-AltPrP antibody was validated in Western blot experiments using lysates from untransfected (mock) HEK293 cells and cells transfected with human PrP or AltPrP. A band was detected with the expected molecular weight in lysates from PrP- and AltPrP-expressing cells (Fig. 6A). The addition of the immunogenic peptide to neutralize the antibodies completely prevented the detection of AltPrP. AltPrP also was not detected with preimmunized serum. To determine the expression of AltPrP from cells expressing endogenous PrP, we used human astrocytoma U-118 cells, which express high levels of PrP. A band was

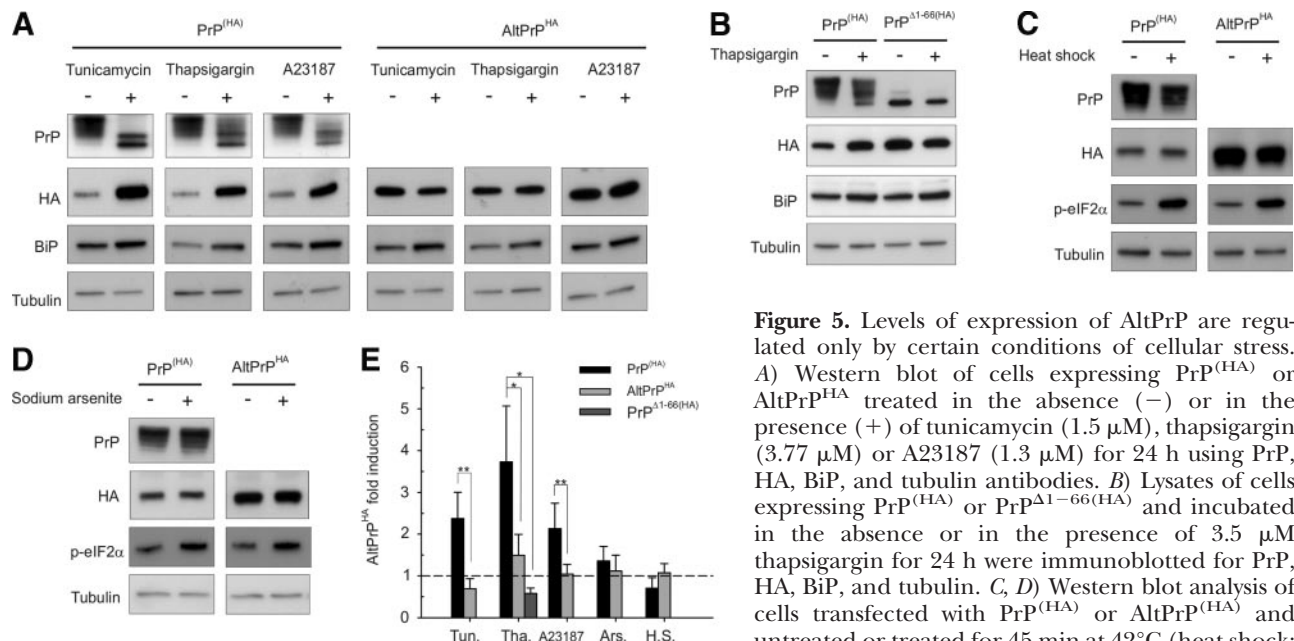


Figure 5. Levels of expression of AltPrP are regulated only by certain conditions of cellular stress. **A)** Western blot of cells expressing PrP^(HA) or AltPrP^{HA} treated in the absence (–) or in the presence (+) of tunicamycin (1.5 μ M), thapsigargin (3.77 μ M) or A23187 (1.3 μ M) for 24 h using PrP, HA, BiP, and tubulin antibodies. **B)** Lysates of cells expressing PrP^(HA) or PrP^{Δ1–66(HA)} and incubated in the absence or in the presence of 3.5 μ M thapsigargin for 24 h were immunoblotted for PrP, HA, BiP, and tubulin. **C, D)** Western blot analysis of cells transfected with PrP^(HA) or AltPrP^{HA} and untreated or treated for 45 min at 42°C (heat shock; **C**), or for 30 min with 0.5 mM sodium arsenite (**D**) with anti-PrP, anti-HA, anti-phospho-eIF2 α (p-eIF2 α), and anti-tubulin antibodies. Cells were allowed to recover for 8 h before harvesting. **E)** Densitometric quantification of AltPrP^{HA} induction under several cellular stress conditions. Tunicamycin (Tun.), thapsigargin (Tha.), and A23187 each significantly induced AltPrP^{HA} expression from PrP^(HA) as compared to the AltPrP^{HA} construct. Stimulation of AltPrP expression in the presence of thapsigargin was abolished by the deletion of bp 1–66 relative to translation initiation site of PrP (PrP^{Δ1–66(HA)}). Control experiments using cells transfected with PrP^(HA) and treated with heat shock (H.S.) or sodium arsenite (Ars.) did not produce significant changes in AltPrP^{HA} expression levels. Dotted line represents AltPrP^{HA} levels in untreated transfected cells normalized to 1. Values are expressed as means \pm sd from ≥ 3 independent experiments. * $P < 0.05$, ** $P < 0.01$; t test.

detected at the expected size in U-118 whole-cell lysates, suggesting that endogenous AltPrP is, indeed, detected by the antibody (Fig. 6B). The identity of AltPrP was confirmed by treating the cells with siRNA against *PRNP*. Western blot analysis proves that AltPrP is endogenously expressed in these cells, since *PRNP* knockdown resulted in a 60% decrease of the intensity of the bands corresponding to both PrP and AltPrP (Fig. 6B, C). Furthermore, mitochondrial fractionation shows that, like AltPrP^{HA}, the band corresponding to AltPrP is enriched in mitochondria, and is absent in the cytosolic fraction (Fig. 6D). Notably, expression of endogenous AltPrP was up-regulated following treatment with thapsigargin, further indicating that the level of AltPrP expression might be increased during neurodegeneration (Fig. 6E, F). Following this experiment, primary human cells that express PrP were tested for AltPrP expression using the same antibody. In addition to U-118 cells, human primary neurons, as well as human peripheral blood mononuclear cells, express AltPrP, judging by the prominent band at the same molecular weight as the band present in a control cell lysate transfected with AltPrP (Fig. 6G). Furthermore, a healthy human brain homogenate was tested for AltPrP expression using the same technique. As expected, the homogenate expressed a band at the same molecular weight (Fig. 6H).

Overall, these results clearly establish that, in different tissues, primary cells, and cell lines, two protein products, PrP and AltPrP, are endogenously expressed from two distinct overlapping reading frames present in the *PRNP* gene.

DISCUSSION

In this study, we provide evidence that out-of-frame alternative translation initiation in the human, sheep, bovine, and deer *PRNP* gene, which encodes the cellular prion protein, results in the synthesis of a novel polypeptide that we termed AltPrP. In-frame alternative translation in PrP mRNA has been reported to produce N-terminally truncated cytoplasmic or nucleocytoplasmic forms of hamster, human, and sheep PrP, which might have a different function from that of GPI-anchored PrP (33, 34). Here, we propose that coexpression of AltPrP along with PrP represents an additional level of complexity regarding the functions attributed to the *PRNP* locus. The production of several polypeptides from this single gene, with modulation depending on cellular conditions, represents a very plausible explanation for the difficulty in assessing the physiological function of PrP, and more generally of the *PRNP* locus. Knockdown or knockout of PrP expression by targeted inactivation of PrP mRNA or the *PRNP* gene most likely results in knockdown of all PrP isoforms as well as AltPrP (Fig. 6C). To get insights into the molecular mechanisms of PrP function, these considerations should be taken into account in the interpretation of experimental results in the future.

The investigation of AltPrP functions is likely to shed some light on the physiological role of PrP, as controversies have emerged in the literature (35–37). We propose that part of the difficulty in assessing the physiological relevance of the *PRNP* gene could be

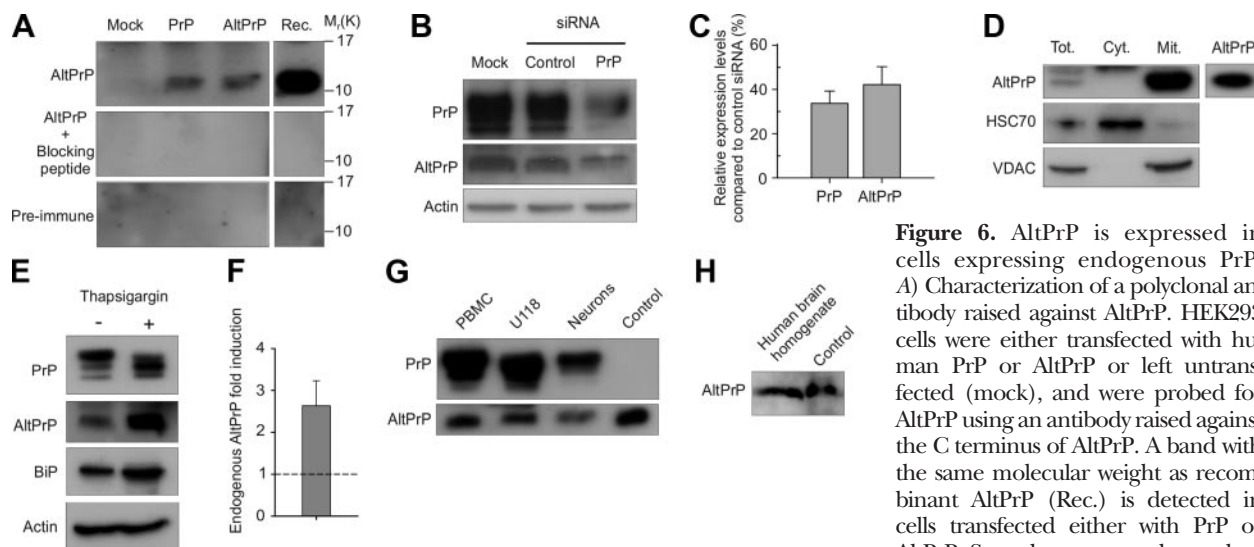


Figure 6. AltPrP is expressed in cells expressing endogenous PrP. *A*) Characterization of a polyclonal antibody raised against AltPrP. HEK293 cells were either transfected with human PrP or AltPrP or left untransfected (mock), and were probed for AltPrP using an antibody raised against the C terminus of AltPrP. A band with the same molecular weight as recombinant AltPrP (Rec.) is detected in cells transfected either with PrP or AltPrP. Same lysates were also probed

with the anti-AltPrP antibody blocked with the immunogenic peptide against which the antibody was targeted or with the animal's preimmunized serum in order to demonstrate the specificity of the antibody. *B*) Endogenous AltPrP was detected in U-118 cells, which express high levels of PrP. An siRNA against the 3'UTR of *PRNP* (PrP) notably reduced the expression of PrP, as well as the band corresponding to AltPrP at comparable levels, whereas a control siRNA did not change PrP and AltPrP expression. *C*) Densitometric analysis of siRNA treatment revealed that U-118 cells transfected with an siRNA against PrP showed a decrease (60–70%) in expression of both PrP and AltPrP following treatment. No statistically significant difference was observed between the knockdown levels of PrP and AltPrP by paired *t* test ($n=2$). *D*) Total cell extracts (Tot.), mitochondrial (Mit.), and cytoplasmic (Cyt.) fractions from U-118 cells were immunoblotted for AltPrP; VDAC, a mitochondrial marker; and HSC70, a marker of the cytosol. Band corresponding to AltPrP was enriched in mitochondria, as expected. AltPrP represents a lysate from cells transfected with AltPrP and is used as a control. *E*) Western blot of human astrocytoma U-87 cells expressing endogenous PrP treated in the absence (–) or in the presence (+) of thapsigargin (3.77 μ M) for 24 h using PrP, AltPrP, BiP, and actin antibodies. *F*) Densitometric quantification of endogenous AltPrP induction in the presence of thapsigargin. Dotted line represents AltPrP levels in untreated U-87 cells normalized to 1. Values are expressed as means \pm SD from ≥ 4 independent experiments. *G*) Detection of endogenous AltPrP in several cell types. Human peripheral blood mononuclear cells (PBMCs), U-118 cells, and human primary neurons (neurons) were immunoblotted for both PrP and AltPrP. All cell types tested showed a prominent band at the same molecular weight as a positive control consisting of cells transfected with human AltPrP (control). *H*) Human brain homogenate was probed for AltPrP using the anti-AltPrP antibody. A band at the same molecular weight as the one seen in cells transfected with human AltPrP was detected (control).

attributed to the coexpression of AltPrP together with PrP from the *PRNP* gene. For instance, the neuroprotective role of PrP has been largely debated and seems to be context dependent (38, 39). Because AltPrP is localized at the mitochondria (Fig. 3), an essential organelle for energy metabolism, stress response, and apoptosis (among other functions), it is tempting to think that some toxic or protective functions have been misattributed to PrP. Though these speculations remain to be proven, we have provided evidence that support this hypothesis. The increased level of AltPrP synthesis under conditions of ER stress (Fig. 5) represents a characteristic feature of various proteins implicated in the unfolded protein response that participate in stress recovery or, if cellular damage is irreversible, induction of apoptosis (30). It is to be noted that AltPrP does not seem to be proapoptotic on its own, since we were able to maintain cell lines stably overexpressing AltPrP^{HA} without any noticeable change in growth rate or cellular morphology (data not shown). However, one can imagine that AltPrP might participate in either a proapoptotic or antiapoptotic response under specific cellular conditions.

An interesting consideration regarding the discovery of AltPrP is its high degree of conservation and homol-

ogy in a large number of mammals (Fig. 1). Although numerous studies have demonstrated the conservation of the C-terminal portion of PrP among different species, explained by the highly structured nature of this domain, as well as its necessity for forming PrP^{Sc} (40, 41), there still lacks a convincing argument explaining why the unstructured N-terminal domain of PrP is conserved as well (40, 42–44). Despite the fact that the function of AltPrP remains unknown, this newly discovered protein may be the reason for the conservation of the N-terminal portion of PrP. Selective pressure might act on the *Prnp* gene in order to conserve AltPrP rather than the unstructured fragment of PrP. At this point, this is merely a speculation, but it provides another compelling reason to continue studying both the *Prnp* gene and AltPrP.

The role of PrP in the pathogenesis of TSEs is very well established, as self-templated transition of the native isoform of PrP (PrP^C) to its misfolded, disease-linked isoform (PrP^{Sc}), is at the origin of the neurotoxic mechanisms implicated in these neurodegenerative disorders. As a result, the *PRNP* locus is the main genetic risk factor in these diseases (45). Since this gene also directs the synthesis of AltPrP, the possibility exists that this novel polypeptide might be linked to

TSEs. AltPrP could be functionally implicated in TSEs, although our data suggest that basic features of PrP biology, including glycosylation, localization, and solubility, do not seem to depend on coexpression of AltPrP. Our results are based on a cell culture system, a model that does not reproduce the events taking place at the level of a whole organism during disease. Moreover, it is plausible that the currently unknown function of AltPrP will have no direct effect on PrP biology. Nevertheless, the possibility that AltPrP might participate in the pathogenesis of TSEs deserves further investigation, and determining its function will likely answer this question. Regardless of whether AltPrP will be proven to have a functional relationship with the pathogenesis of TSEs, the fact that its expression is greatly enhanced following proteasome inhibition and ER stress (Figs. 4 and 5) makes it a potential biomarker for neurodegenerative disorders. Indeed, these two cellular stresses have been shown to be hallmarks not only of TSEs, but also of other neurodegenerative diseases, such as Huntington's, Parkinson's, and Alzheimer's diseases (27, 28, 46).

Other examples of polypeptides synthesized from out-of-frame downstream alternative translation initiation sites exist in the literature, although this mechanism occurs most often in viruses (47–49). Very few mammalian examples of this phenomenon have so far been described (12, 13). Klemke and colleagues (12) have discovered and characterized ALEX, a protein encoded by an alternative ORF in the *XLAs/Gas* gene and that is expressed *in vivo* in both rats and humans. Cryptic T-cell epitopes represent other examples of this phenomenon in humans. However, except for their immunogenic potential, these polypeptides have not been further characterized (50). To our knowledge, AltPrP represents the second extensively characterized product of out-of-frame alternative translation initiation in a human gene. The discovery of AltPrP could not only change our understanding of the *PRNP* locus, but the fact that AltPrP is endogenously expressed supports the idea that out-of-frame alternative translation initiation in mammals is very likely to participate in protein diversity. This reveals an additional level of complexity to the proteome and to gene function, which deserves further investigation in order to determine its physiological importance. FJ

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en Santé du Québec. Nucleotide sequence data reported in Fig. 1 are available in the Third Party Annotation Section of the DDBJ/EMBL/GenBank databases under accession numbers TPA: BK007887–BK007890.

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