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LIV-1 ZIP Ectodomain Shedding in Prion-Infected Mice Resembles Cellular Response to Transition Metal Starvation

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Abstract

We recently documented the co-purification of members of the LIV-1 subfamily of ZIP (*Zrt*-, *Itt*-like *Protein*) zinc transporters (LZTs) with the cellular prion protein (PrP^C) and, subsequently, established that the prion gene family descended from an ancestral LZT gene. Here, we begin to address whether the study of LZTs can shed light on the biology of prion proteins in health and disease. Starting from an observation of an abnormal LZT immunoreactive band in prion-infected mice, subsequent cell biological analyses uncovered a surprisingly coordinated biology of ZIP10 (an LZT member) and prion proteins that involves alterations to N-glycosylation and endoproteolysis in response to manipulations to the extracellular divalent cation milieu. Starving cells of manganese or zinc, but not copper, causes shedding of the N1 fragment of PrP^C and of the ectodomain of ZIP10. For ZIP10, this posttranslational biology is influenced by an interaction between its PrP-like ectodomain and a conserved metal coordination site within its C-terminal multi-spanning transmembrane domain. The transition metal starvation-induced cleavage of ZIP10 can be differentiated by an immature N-glycosylation signature from a constitutive cleavage targeting the same site. Data from this work provide a first glimpse into a hitherto neglected

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molecular biology that ties PrP to its LZT cousins and suggest that manganese or zinc starvation may contribute to the etiology of prion disease in mice.

Keywords

endoproteolysis; evolution; prion proteins; zinc; ZIP proteins

Introduction

The prion protein (PrP) plays an essential role in the occurrence of the fatal neurodegenerative prion diseases of humans and animals, which can manifest through sporadic, familial or infectious etiologies.^{1,2} In prion disease, the normal cellular prion protein, denoted PrP^C, undergoes a structural transition to its disease-causing PrP^{Sc} form (PrP^{Sc}) with profoundly different physicochemical properties. Mice devoid of PrP^C are largely indistinguishable from wild-type mice, an observation that has hampered efforts to uncover the cellular function of PrP^C.³ Similarly, parallel attempts to dissect the role of PrP^C in cell biological studies have frequently led to inconclusive and, at times, contradictory results.⁴ Investments in both approaches, however, have not been futile. Persistent efforts to tease out phenotypic abnormalities in knockout mice, for example, have recently led to the identification of PrP^C as a contributor to a myelin maintenance phenotype in the peripheral nervous system.⁵ Cell biological studies, on the other hand, have generated a body of literature that points at a role of PrP^C in cellular metal homeostasis and/or the protection of cells against oxidative insults.⁶ A third strand of investigations aimed at revealing the cellular function of PrP^C by studying its molecular interactions has similarly led to no shortage of candidate interactors, some of which have been independently confirmed by multiple research groups. In one such PrP^C interactome study, which was based on *in vivo* cross-linking of murine neuroblastoma (N2a, Neuro-2a) cells followed by quantitative mass spectrometry, we recently identified members of the ZIP (*Zrt*-, *Irt*-like *Protein*) metal ion transporter family in proximity to the prion protein.⁷ Surprisingly, extensive bioinformatic analyses subsequently established an evolutionary link between the prion gene and the ancient family of ZIP transporters.⁸ More precisely, our analyses established a shared ancestry between the prion gene and a phylogenetic branch of ZIP transporters, which is also referred to as the LIV-1 subfamily of ZIP zinc transporters (LZTs). Sequence similarities and structural threading analyses led to the prediction that a subset of LZT paralogs are equipped with PrP-like N-terminal ectodomains, which protrude into the extracellular/luminal space and harbor a cysteine-flanked core (CFC) domain that is homologous to the respective CFC domains observed in contemporary prion proteins. With regard to the likely mechanism of evolution, the most parsimonious interpretation of our current genomic data suggests that, 0.5 billion years ago, a genomic insertion of a spliced transcript coding for a PrP-like ZIP ectodomain may have created the prion founder gene in a vertebrate ancestor.^{9,10} On the basis of sequence similarities, it is likely that this LZT ancestor belonged to a phylogenetic subbranch that, in contemporary species, is populated by ZIP5, ZIP6 and ZIP10. It might be instructive to understand why two of the three members of this subbranch of LZTs, namely ZIP6 and ZIP10 but not ZIP5, co-purified with PrP^C in the aforementioned interactome study. In contrast to the vast literature surrounding the biology of PrP, little is

known about the LZTs. This knowledge gap pertains to many aspects of ZIP biology, including their expression, posttranslational modifications, molecular structure, regulation and response to divalent cations. Assuming that some overlaps in the biology of LZT paralogs exist, a close examination of ZIP4 may be warranted in efforts to predict the biology of ZIP5, ZIP6 and ZIP10. ZIP4 has been genetically linked to an autosomal recessive zinc deficiency disease in children, termed acrodermatitis enteropathica, and has been subjected to closer scrutiny than other LZTs as a consequence. Biochemical studies of ZIP4 suggest that its expression inversely correlates with extracellular zinc levels.^{11,12} Furthermore, it has been shown that a large part of what we termed the PrP-like ectodomain of ZIP4 can be shed by an endoproteolytic cleavage event in healthy individuals but, possibly, not in a subset of individuals suffering from acrodermatitis enteropathica.¹³ Interestingly, this shedding seems to occur in response to zinc depletion, suggesting that it may constitute a mechanism by which the cell can counteract undesired zinc starvation stresses.

The objective of this work was to complement our previous interactome analyses (which identified LZTs as candidate PrP^C interactors) and bioinformatic investigations of the relationship between prion and LZT genes, with experiments that explore a possible involvement of LZTs in the normal and disease biology of the prion protein. We document that, in mice challenged with mouse-adapted Rocky Mountain Laboratory (RML) prions, the generation of a novel ZIP10 immunoreactive band can be observed from as early as 84 days after intracerebral (i.c.) inoculation. Subsequent experiments aimed at characterizing this ZIP10-derived signal uncovered a surprising degree of crosstalk in the biology of ZIP10 and prion proteins and shed light on a complex biology surrounding N-glycosylation and the generation of endoproteolytic cleavages within the PrP-like domains of ZIP10 and PrP that is tied to cellular divalent cation homeostasis. We discuss the relevance of our data to understanding the functional role of an α -site cleavage observed in PrP^C and propose that prion disease in mice might phenocopy a transition metal starvation phenotype.

Results

Prion disease in mice is accompanied by abnormal endoproteolysis of ZIP10

Whereas comprehensive time-course data that document changes to the global transcriptome in response to challenging mice with prions exist,¹⁴ a similar analysis at the proteome level is lacking. Of note, however, is a study that uncovered a robust and progressive reduction in protein but not mRNA levels of the prion paralog Shadoo during prion disease manifestation.¹⁵ In light of these earlier observations, we wondered whether protein levels of LZTs might be similarly modified in mice, which had been intracerebrally inoculated with mouseadapted prions derived from the RML prion strain. Because ZIP10 was predicted to be expressed in the brain¹⁶ and was identified as a candidate interactor of PrP^C in N2a cells, we set out to raise a polyclonal antibody directed against a paralog-specific epitope within the (N-terminal) ectodomain of endogenous mouse ZIP10 (anti-ZIP10_N). Subsequent immunoblot analyses following the separation of mouse brain extracts by denaturing SDS-PAGE confirmed the expression of ZIP10 in the brain (Fig. 1). The band pattern revealed by the ZIP10-directed antibody, however, was complex, suggesting that the

antibody may cross-react with other proteins and/or the respective endogenous ZIP protein may undergo complex posttranslational modifications (please see below).

A comparison of the ZIP10 band pattern observed in control mice and littermates 84 days post-inoculation (p.i.) with RML prions appeared largely identical. Strikingly, however, the analysis of brain extracts of mice sacrificed 131 days p.i. with prions exhibited an additional band that migrated with an apparent molecular weight (MW) of 45 kDa (anti-ZIP10_N). In fact, this band was already detectable 84 days p.i. and was much less pronounced in age-matched control animals that had been inoculated with brain extracts from naïve mice. When probed with an antibody (Sha31) that recognizes endogenous PrP, the 131 p.i. RML-inoculated brain extract revealed the expected aggregation of PrP characteristic of prion-infected mice. Parallel comparisons we undertook with antibodies that recognize iron or copper transporters [TfR (*transferrin receptor*) or CCS (*copper chaperone for superoxide dismutase*)] or can probe for the expression of GAPDH (*glyceraldehyde 3-phosphate dehydrogenase*) documented no differences in protein levels or posttranslational modifications of these control proteins between non-inoculated and RML-inoculated mice. When we probed additional cell status reporter proteins that can inform about synaptic integrity (Drebrin and Munc18) or cellular stress (MnSOD and Histone H4), subtle age-related differences in their expression levels were observed, but again, no significant difference in age-matched RML-inoculated *versus* control brains were seen (Fig. S1). A subsequent repetition of the entire experiment led to an identical result and thus ruled out that these observations represent mere idiosyncrasies of the first cohort of mice. Taken together, these experiments suggested that the manifestation of prion disease in mouse brains might be paralleled by alterations in the expression or posttranslational modification of the ZIP10 transporter that populates the evolutionarily nearest phylogenetic subbranch of LZTs. However, this experiment also raised many questions that might be answered best by moving to an experimental paradigm more amenable to experimental manipulation.

ZIP10 is one of many LZTs expressed in N2a cells that localizes to the plasma membrane and undergoes complex posttranslational maturation

Of the nine LZTs present in the genomes of mice and humans, up to seven may contain PrP-like ectodomains.⁹ In preparation for studies in the mouse neuroblastoma cell model N2a, the best-characterized cell model for the study of prion biology to date, reverse transcription polymerase chain reactions (PCRs) comparing the transcriptional expression of relevant LZTs in N2a cells and brains of 12-week-old CD-1 outbred mice were conducted. Data from this experiment suggested that a complex LZT biology may exist in N2a cells and that this cell model may constitute a suitable proxy to the brain for studying the biology of LZTs (Fig. S2). Furthermore, it provided a straightforward explanation for why ZIP5, the LZT paralog that has retained the strongest sequence similarity to its molecular cousin PrP^C, could not, unlike ZIP6 and ZIP10, be identified as a PrP^C candidate interactor in our interactome study that was also based on N2a cells.⁷

We next conducted confocal immunofluorescence analyses of hemagglutinin (HA)-tagged ZIP10 in N2a cells and observed the expected predominant localization of this protein at the plasma membrane, with signals primarily appearing in the form of distinct puncta and

occasionally extending into filamentous membrane protrusions (Fig. 2b). Additional punctate intracellular signals but no nuclear signals were observed, consistent with a cell biology that involves passage through the secretory pathway and possible degradation in endolysosomal compartments. These data were in good agreement with immunohistochemical data we collected with the in-house-generated antibody recognizing ZIP10 (Fig. 2a and c). The latter, however, showed less intracellular staining, conceivably a consequence of a lower turnover rate of ZIP10 in mature brain relative to cells grown in culture. Next, co-immunofluorescence analyses of the same HA-tagged ZIP10 and endogenous PrP^C were undertaken, revealing that, at most, a partial co-localization of PrP and ZIP10 may exist in cells (Fig. S3a). Similarly, no ZIP10 was detected in the low-density sucrose fraction (lipid rafts) populated by PrP^C that is known to be enriched in cholesterol and sphingolipids and can be obtained following extraction of ZIP10-HA-expressing cells in cold Triton X-100 (Fig. S3b). These data were consistent with the low ZIP10 sequence coverage observed in the PrP^C interactome analysis that preceded this work,⁷ a possible indicator that either the interaction with the PrP^C bait was relatively weak or only a small population of the total cellular pools of PrP^C and ZIP10 proteins engage in protein–protein interactions.

A wealth of data characterizing the molecular biology of PrP^C and its posttranslational processing in N2a cells exists. To begin understanding the significance of the ZIP10–PrP relationship at the molecular level, a biochemical characterization of ZIP10 in N2a cells might be informative. When analyzed by denaturing SDS-PAGE, the heterologous expression of ZIP10 in N2a cells caused the appearance of multiple bands (Fig. 2d). Prominent signals detected migrated with apparent MWs of 80–90 kDa, consistent with the expected size of full-length ZIP10. Additional lower MW bands were observed in the 40–60 kDa range. Initially, the ZIP10-HA expression construct was separately analyzed with a monoclonal antibody that detects the HA-tag at the C-terminus of ZIP10-HA or the aforementioned in-house-generated polyclonal antibody (used in Fig. 1) that recognizes an epitope present within the ectodomain of both endogenous ZIP10 and overexpressed ZIP10-HA (Fig. 2a). This analysis revealed a band pattern for endogenous ZIP10 in the non-transfected (empty plasmid) control that was qualitatively identical with the one observed following ZIP10 overexpression, thereby establishing that the appearance of the band duplet, which we interpreted to represent full-length ZIP10, did not constitute an overexpression artifact. On the basis of published work documenting N-glycosylation of a subset of LZTs^{17,18} and the presence of “N_xT” consensus motifs within the amino acid sequences of ZIP4, ZIP5 and ZIP10 (Fig. S4), it seemed likely that N-glycosylation also contributed to the complexity of the band patterns observed in these experiments. Indeed, PNGase F digestion of cell extracts harvested following ZIP10-HA transfection caused the full-length ZIP10-HA band duplet to be replaced with a single band (Fig. 2e). These data established ZIP10 to be partially posttranslationally modified by N-glycosylation and to be subject to endoproteolysis. On the other hand, alternative splicing was unlikely to be a major factor contributing to ZIP10 band complexity because the expression of ZIP10 from a heterologous cassette vector that lacked the genomic intron/exon structure gave rise to all major bands seen with endogenous ZIP10.

ZIP10 is N-glycosylated at “NxT” acceptor site that it shares with PrP^C

To determine whether N-glycosylation of ZIP10 occurs at the “NxT” acceptor site conserved between PrP and ZIP108 (Fig. S4), a ZIP10-HA_N341A expression construct was generated, which replaced the critical asparagine within the N-glycosylation consensus motif with an alanine. Additional ZIP10-HA-derived truncation constructs were generated to define endoproteolysis sites (Fig. 3a). The rationale for choosing truncation sites followed the anticipation that the CFC domain (the most conserved stretch of sequence in multiple alignments of PrP and LZT sequences)⁸ was unlikely to be targeted by endoproteolysis and that, instead, cleavage sites may flank this domain. Heterologous expression of these constructs in N2a cells followed by immunoblot analyses and PNGase F treatment (Fig. 3b) allowed several conclusions, which established that (i) the N341 N-glycan acceptor site (positionally shared with PrP) is indeed quantitatively N-glycosylated, that (ii) additional N-glycosylations occur within the ectodomain but not within the C-terminal transmembrane domain and that (iii) endoproteolytic cleavages indeed occur at sites flanking the CFC and give rise to C-terminal fragments with approximate N-terminal boundaries near amino acids 300 and 400 (numbering based on wild-type mouse ZIP10), hereafter referred to as ZIP10 C1 and ZIP10 C_ecto, respectively.

Appearance of truncated and N-glycosylated variants of ZIP10 depends on extracellular divalent metal ion status

In preparation for experiments addressing whether metal-induced cleavages of LZTs contribute to the appearance of faster-migrating bands observed following inoculation of mice with RML prions and/or during SDS-PAGE analysis of LZTs in N2a cells, the quantity of divalent metals in the N2a growth medium was measured by inductively coupled plasma atomic emission spectroscopy. This analysis revealed low micromolar zinc levels, 10-fold lower copper levels and negligible manganese levels in the minimal essential medium supplemented with fetal bovine serum used for culturing N2a cells (Table S1). Next, levels of zinc, copper or manganese were experimentally augmented or suppressed in the growth medium by adding the respective salts or including the transition metal chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) (Fig. 4a). Interestingly, the individual bands within the full-length ZIP10 band duplet were affected by these treatments differently; that is, when transition metals were replete, levels of the N-glycosylated derivative of the full-length ZIP10 band were slightly reduced. In contrast, and reminiscent of what had been described for ZIP4,¹³ starving cells of transition metals by chelation resulted in an increase in the intensity of the signal interpreted to represent N-glycosylated full-length ZIP10 and the appearance of a smaller ZIP10 product that migrated at an apparent MW of 45 kDa. Interestingly, the chelation of transition metals with TPEN also caused changes in the way PrP^C-reactive signals were presented when Western blots were immunoprobed with the Sha31 antibody. More specifically, a considerable proportion of full-length N-glycosylated PrP^C had been replaced with Sha31-reactive bands, which migrated at lower apparent MWs, including signals at 19 kDa, and as such lower than the expected MW of unglycosylated full-length PrP^C. This result suggested that treatment of cells with TPEN causes profound changes to posttranslational modifications of PrP^C that include its endoproteolysis. Thus, endogenous ZIP10 and PrP^C undergo proteolysis in the presence of TPEN, and both the

inoculation of mice with mouse-adapted RML prions and TPEN-based chelation can generate a product of endogenous mouse ZIP10 that migrates at 45 kDa.

Appearance of truncated ZIP10 can be rescued by manganese and zinc but not copper replenishment

Given that TPEN has been shown to pass the cellular membrane¹⁹ and, in our hands, was toxic to N2a cells after extended incubation periods (even at low micromolar concentrations), we replaced TPEN in subsequent experiments with the cell-impermeable transition metal chelator diethylenetriamine pentaacetic acid (DTPA), which exhibited lower cytotoxicity than TPEN (Fig. 4b). Importantly, the replacement of TPEN with DTPA did not abolish the generation of the 45-kDa endogenous mouse ZIP10 product or the formation of the 17-kDa PrP-derived signal, indicating that the appearance of these bands is indeed most likely a consequence of N2a cells sensing an extracellular environment deprived of transition metals rather than a result of TPEN exerting this effect independent of its metal chelation properties or on the basis of its capacity to permeate into cells. To explore whether the 45-kDa fragment is generated as a by-product of an intracellular ZIP10 degradation pathway or is shed/secreted into the medium, we repeated the experiment with N2a cells grown in serum-free medium (Fig. 4c). The side-by-side Western blot analysis of an aliquot of the cell culture supernatant of cells grown in the presence of DTPA next to cellular extract fractions from the same cells, followed by densitometric analysis of signal intensities, revealed that approximately 95% of the 45-kDa product was indeed detected in the medium. Since neither TPEN nor DTPA possesses exquisite selectivity for a particular transition metal, experiments up to this point had not answered which of the transition metals, when depleted, caused the appearance of the 45-kDa ZIP10 band. Rescue experiments conducted by adding DTPA together with zinc, copper or manganese to the growth medium established that 25 μ M concentrations of zinc or manganese but not copper can prevent the formation of the 45-kDa ZIP10 band (Fig. 4d). Of note, the functional depletion of transition metals from the medium by the addition of DTPA was again paralleled by a diminution of full-length PrP^C levels. Strikingly, the activity profile of transition metals that enabled rescue of this PrP^C effect was also identical with the profile we had observed for rescuing ZIP10 endoproteolysis; that is, the addition of copper had no influence on the DTPA-mediated diminution of full-length PrP^C signals, despite the ability of DTPA to also chelate copper²⁰ and a well-documented preferential association of PrP^C with copper. Finally, to assess whether another well-established cell model for the study of prion biology responds similarly to the starvation of transition metals, we grew GT1 cells (immortalized murine hypothalamic neurons)²¹ in the presence of DTPA, and again, we analyzed cellular extracts by immunoblotting with anti-ZIP10 or anti-PrP antibodies (Fig. 4e). The analysis gave rise to less complex immunoblot band patterns for both ZIP10 and PrP^C, consistent with the interpretation that endoproteolytic activities are less pronounced in these cells or that proteolytic fragments are more effectively cleared. However, the overall trends with which full-length ZIP10 or PrP^C bands were altered in response to DTPA, as well as the ability of zinc to rescue this phenotype, were not only retained but also appeared even more pronounced. Taken together, these experiments established that starving the cell culture medium of zinc or manganese not only triggers an increase in full-length N-glycosylated ZIP10 levels and the shedding of a 45-kDa ZIP10 ectodomain fragment but also causes a diminution of full-length N-

glycosylated PrP^C, possibly through an endoproteolysis/degradation step. Experiments up to this point, however, failed to reveal the identity of the respective ZIP10 or PrP^C cleavage products and did not address whether a putative zinc metalloproteinase active center present in LZTs may contribute to endoproteolytic cleavages observed.

A zinc coordination site within TMD5 regulates N-glycosylation and endoproteolysis of ZIP10

Sequence alignments of LZTs revealed a conserved “(C/L)(H/E)E(L/I/V/F)PHE(L/I/V/M)GDF” sequence motif within their predicted TMD5 (*transmembrane domain 5*) (Fig. 5a). The conservation of this motif had also been noted by others,²² and two separate roles of histidine residues embedded within this motif were considered: (i) a function in the gating of transition metals or (ii) a contribution to the assembly of a zinc metalloproteinase active center with resemblance to metzincin or deformylase group metalloproteinases.²³ Hypothesizing that the presence of TPEN or DTPA may have interfered with the transition metal occupancy of this coordination center and that one may gain more insight into the identity of the 45-kDa cleavage band by close examination of the role of these histidines, we employed site-directed mutagenesis to generate alanine replacement mutants. The comparison of transiently expressed wild-type ZIP10-HA and mutant ZIP10-HA-(H711A/H715A) by immunoblotting revealed two striking differences: (i) a larger proportion of full-length ZIP10-HA-(H711A/H715A) migrated at the level of N-glycosylated wild-type ZIP10-HA, suggesting that point mutations either altered SDS-PAGE separation characteristics or that the mutant is indeed more readily glycosylated as it traverses the secretory pathway, and (ii) expression of ZIP10-HA-(H711A/H715A) caused the appearance of conspicuous faster-migrating bands. These bands were detected with the polyclonal antibody that targets the ZIP10 ectodomain and migrated in SDS-PAGE analyses with apparent MWs of 50 and 52 kDa (Fig. 5b).

We next investigated the relationship between the faster-migrating bands observed with either TPEN-/DTPA-treated endogenous ZIP10 or mutant ZIP10-HA-(H711A/H715A). Whereas the former appeared to migrate at 45 kDa and the mechanism of its generation was not immediately apparent, the latter was observed as a duplet of 50 and 52 kDa and had to be an endoproteolysis product because it was generated from a plasmid-encoded parent molecule. Interestingly, the DTPA-mediated starvation of transition metals in the culture medium of cells expressing ZIP10-HA-(H711A/H715A) caused these cells to override the molecular biology which led to the 50/52-kDa band duplet, and made the ZIP10-HA-(H711A/H715A)-derived signals look as if they had originated from cells transfected with wild-type ZIP10-HA (Fig. 5c). Thus, the 45-kDa fragment generated by DTPA chelation must itself originate from an endoproteolysis event, which either supersedes cleavages giving rise to the 50/52-kDa band duplet or precedes the formation of the latter within the cellular life cycle of a ZIP10 molecule. Given that the absence of the transition metal coordination center caused a more pronounced N-glycosylation of the parent molecule, it then seemed plausible that the slower migration of the 50/52-kDa band duplet (relative to the 45-kDa band seen with TPEN or DTPA) merely reflected a higher level of N-glycosylation. Indeed, the side-by-side removal of N-linked glycans caused the fast-migrating 50/52-kDa band duplet to be replaced by a single band of 38 kDa, identical in size to the deglycosylated

derivative of the 45-kDa band seen when cells were grown in transition-metal-depleted growth medium (Fig. 5d).

Finally, by further analyzing ZIP10-HA-(H711A/H715A) following the removal of N-linked glycans, it became apparent that the point mutations had indeed caused a larger population of ZIP10-HA-(H711A/H715A) to be N-glycosylated (Fig. 5e). This result was counterintuitive given that N-linked glycans had been mapped to the ectodomain (Fig. 3b), not to the C-terminal transmembrane domains that form the membrane channel of which the mutated sequence motif is a part. This result was illuminating, however, as it suggested that the ectodomain might engage in an interaction that relies on the integrity of the conserved histidines within TMD5. Taken together, experiments in this section revealed the identity of multiple ectodomain fragments observed with different apparent MWs in response to transition metal starvation or mutation of conserved TMD5 histidine residues. It showed that these fragments constitute products of ZIP10 cleavage at a site near ZIP10 amino acid 400 (giving rise to C_ecto and N_ecto products), which merely differ with regard to the level of occupancy of up to four *N*-glycan acceptor sites present within the ectodomain of ZIP10 (Fig. 3a and Fig. S4).

Transition metal starvation or RML infection causes PrP^C to shed distinct N1 or N2 fragments but triggers shedding of identical N_ecto fragments from immature ZIP10

We next turned to characterizing the underlying cause for the diminution of PrP^C levels under manganese or zinc starvation and to understanding the relationship of cleavages observed following infection with prions *versus* starvation of transition metals. It has repeatedly been shown that the prion protein can be cleaved at a so-called α -site bond connecting amino acids 109 and 110 (in mouse),²⁴ causing the release of an N-terminal fragment N1 into the medium and the cellular retention of a C-terminal C1 fragment. A second scissile bond within PrP, termed the β -site, has been identified in proximity to the C-terminal boundary of the octapeptide repeat region.²⁵ Cleavages at the β -site, giving rise to fragments designated as N2 and C2, had been shown to be considerably increased upon prion infection.²⁶ Both C1 and C2 are routinely identified by their characteristic apparent MWs following enzymatic removal of *N*-glycans.²⁷ In this study, the immunoblot analysis of cellular fractions obtained following transition metal starvation *versus* infection with prions demonstrated the former treatment to increase the amount of C1 and confirmed the anticipated increase of C2 in prion-infected cells or mouse brain (Fig. 6a). In contrast, a side-by-side comparison of these extracts with the antibody reactive toward the ZIP10 N-terminus revealed that both transition metal starvation of cells and RML inoculation of mice led to the selective appearance of a ZIP10 fragment that migrates with an identical apparent MW of 45 kDa, which can be reduced to a band migrating at 38 kDa when deglycosylated (Figs. 5d and 6b). On the basis of the analyses presented above, it can be deduced that this band represents a ZIP10 ectodomain (N_ecto) fragment in which only one *N*-glycan acceptor site is occupied. Taken together, these experiments (i) established that α -site cleavages in PrP can be triggered by transition metal starvation and (ii) identified a subset of ZIP10 expression products in prion-infected mice to bear a molecular signature of cellular transition metal starvation.

Discussion

Data presented in this manuscript constitute the first steps toward elucidating the functional significance of the evolutionary relationship between the prion gene and the gene family of ZIP metal ion transporters.^{8,9} Because the primary motivation to conduct this study was to explore whether members of the LIV-1 subfamily of ZIP transporters play a role in prion disease, an observation of abnormal LZT-derived expression products in mice challenged with prion disease served as a starting point for this project. During the course of this work, interesting facets of a hitherto largely unknown biology of LZTs were uncovered, including: (i) the broad expression of many LZTs in N2a cells, (ii) the predominant localization of ZIP10 to the cell surface, (iii) the demonstration of occupancy of up to four N-glycosylation acceptor sites within the ectodomain of ZIP10, (iv) the approximate location of scissile bonds flanking the CFC sequence segment within ZIP10, (v) the finding that zinc or manganese (but not copper starvation and not overabundance of transition metals) causes shedding of the ectodomain of immature ZIP10 into the medium, (vi) evidence that the replacement of conserved histidine residues within TMD5 but not the addition of a C-terminal HA-tag interferes with the activity of ZIP10 and (vii) the observation that both the growing of cells in medium starved of transition metals and the expression of the ZIP10 construct harboring TMD5 histidine replacement mutations cause cleavage of the same ZIP10 scissile bond, with the consequence that the bulk of ZIP10 PrP-like ectodomains are released into the medium (Fig. 6c and d). Taken together, these data combine to a model whereby cells grown in standard medium express primarily full-length ZIP10, which is gradually N-glycosylated, reaching a maximum occupancy when all four “N_xT” ectodomain acceptor sites carry *N*-glycans. Expression levels of ZIP10 are posttranslationally controlled by the cell, possibly to avoid toxic intracellular transition metal levels from accumulating through ZIP10-dependent passive diffusion [it should be noted that regulation at the transcriptional level seems implausible because ZIP10-HA and ZIP10-HA-(H711A/715A) were shown in this work to express at very different levels despite their transcription being driven from identical heterologous expression plasmids] (Fig. 5c). Removal of a highly conserved zinc coordination center in TMD5 generates a non-gating ZIP10 molecule, which is no longer subject to the same posttranslational degradation processes and therefore accumulates to higher protein levels. A higher N-glycosylation level and increased ectodomain shedding of this ZIP10 mutant suggests that the ZIP10 ectodomain containing both the four “N_xT” acceptor sites and the scissile bond for endoproteolysis might engage in a transition metal-dependent interaction with TMD5 in wild-type ZIP10 but not in ZIP10-(H711A/H715A). When the cell culture medium is starved of transition metals, the first line of response by the cells is to slow the degradation of full-length N-glycosylated ZIP10, which not only leads to a stronger signal for the corresponding Western blot signal derived from endogenous ZIP10 but also causes the heterologous expression of wild-type ZIP10-HA to proceed to higher steady-state levels of the mature ZIP10 protein. The low transition metal influx that is likely experienced by cells starved of manganese or zinc further causes newly made ZIP10 ectodomains to be shed at a time when only one *N*-glycan has been transferred to its four eligible “N_xT” acceptor sites. The i.c. challenge of mice with mouse-adapted RML prions causes a subpopulation of ZIP10 to acquire the molecular characteristics of ZIP10 molecules grown in cell medium starved of transition metals.

With the current data, it cannot be resolved whether the shedding of ZIP10 ectodomains and N-terminal PrP^C fragments constitute independent responses to the same extracellular stimulus or rely on more immediate crosstalk between LZTs and PrP^C. In particular, the transition metal starvation data presented in this work either could represent the manifestation of independent cellular pathways that share a reliance on a transition metal starvation stimulus or may reflect direct crosstalk and a shared molecular biology of LZTs and PrP^C. Favoring the latter interpretation are observations documenting that (i) ZIPs and PrP^C can co-immunoprecipitate (demonstrated in work that preceded this study for ZIP6/ZIP10),⁷ that (ii) RML infection of mice causes the abnormal appearance of ZIP10-derived products as early as 84 days following i.c. challenge (i.e., at a time during disease progression when other molecular defects are not yet pronounced and no overt symptoms can be detected in mice) and that (iii) a striking correlation exists in the response of ZIP10 or PrP^C to transition metal starvation. This correlation not only pertains to the timing of cleavages and the strength of the response but also extends to the identity of transition metals that, when withdrawn, caused (manganese or zinc) or failed to cause (copper) the shedding of N-terminal fragments from ZIP10 or PrP^C.

Functional significance of ZIP10 ectodomain shedding

The function of what we had termed the PrP-like ectodomain within ZIP10 had not been specifically investigated prior to this study. However, data on the not-so-distant LZT protein family member ZIP4 suggested that its ectodomain may serve a role as a negative regulator or sensor of transition metal uptake. It had also been observed that, under conditions of transition metal starvation, the ZIP4 ectodomain can be shed to overcome the inhibitory influence it exerts on cation uptake.¹³ Data generated in this work support this view and suggest that the proposed inhibitory role may be a more common feature of LZT ectodomains in general. If the ectodomains of LZTs indeed serve an inhibitory function toward the uptake of transition metals, it then follows that the prion founder gene itself, which was evolutionarily descended from an ectodomain segment of an ancestral LZT gene, might have had a biological function in the sensing and negative regulation of transition metal uptake. Is there any hint in the data presented here that could substantiate the possibility that contemporary prion proteins have retained facets of such an inhibitory function? One of the most striking observations made in this study was the diametrical nature in which full-length N-glycosylated ZIP10 and PrP^C signals responded to starving the cell culture medium of GT1 cells of transition metals (Fig. 4e). One way to interpret these observations would be to assume that both the increase in steady-state N-glycosylated full-length ZIP10 levels and the dramatic decrease in mature PrP^C observed under these conditions served a common objective, that is, to respond to the shortage of transition metals in the medium by adjusting the cellular proteome in ways that facilitate transition metal uptake. It can then be deduced that the cellular expression of PrP^C might indeed represent a hindrance to transition metal uptake. Enthusiasm for such a model might be tempered by a body of literature that documents preferential binding of mammalian PrP^C to copper and, only to a much lesser extent, to zinc or manganese. If the ability of PrP^C to coordinate transition metals with the known binding affinities had any connection to its putative inhibitory function, then it would seem counterintuitive that starving cells of manganese or zinc but not copper leads to a diminution of PrP^C levels. One way to reconcile these

seemingly paradoxical observations is to assume that the cell's response to zinc or manganese starvation with regard to PrP^C levels constitutes a remnant from an earlier evolutionary time. According to this model, the transition metal binding profile of ancient prion proteins was dominated by zinc coordination sites. Only gradually, and most pronounced in the tetrapod lineage, prion protein sequences diverged in ways that led to a bias for copper binding. In support of this model, a subset of prion proteins coded for by prion gene orthologs in contemporary fish species still display strong affinity for zinc.²⁸ There is also the possibility that PrP^C may exert an inhibitory effect on zinc or manganese uptake independent of its own transition metal binding profile but on the basis of its ability to bind to a subset of LZTs, which themselves may play a role in zinc or manganese uptake. The demonstration that PrP^C co-localizes and can be immunoprecipitated with a subset of LZTs, together with the observation that highly conserved amino acids within the putative transmembrane domain of LZTs (shown in this work for ZIP10) can influence cleavages and N-glycosylation of their PrP-like ectodomain, strengthen this scenario. It is noteworthy that it is not necessary to invoke that PrP^C represents a critical functional interactor of ZIPs, as it would be sufficient for contemporary PrP^C molecules to have retained a rudimentary ability to engage in contacts with the transmembrane domains of a subset of LZTs. Similarly, the α -site cleavage of PrP^C might, in this scenario, not constitute a “useful” endoproteolysis event in the same sense in which the shedding of the ZIP10 ectodomain from the C-terminal ZIP10 transmembrane domain would be expected to open the passive diffusion channel of this ion transporter for the import of zinc or manganese. Instead, the cleavage of PrP^C at the α -site scissile bond may represent a bystander effect inadvertently directed at PrP^C molecules, which, due to their affinity for a subset of LZTs, can be found enriched within their molecular environment. For this to be a plausible course of events, PrP^C would merely need to have kept enough of its original LZT ectodomain features to still serve as a substrate for an endoprotease, which gets recruited to LZTs to rid them of their inhibitory ectodomains whenever cells are starved of zinc or manganese. Interestingly, a similar course of events might also underlie the posttranslational degradation of the prion family member Shadoo, observed in various strains of mice during the progression of prion disease.²⁹ The cellular compartment in which the LZT ectodomain shedding event occurs is currently not known. With regard to ZIP4 ectodomain shedding, it had been proposed that recycling endosomes may host this cleavage event. Data presented here now suggest that, at least for ZIP10, the shedding of its ectodomain in response to transition metal starvation may selectively affect immature ZIP10 ectodomains. It was further demonstrated that the appearance of the cleaved immature band supersedes the generation of the mature ectodomain fragment observed when ZIP10-HA-(H711A/H715A) is expressed. Taken together, the most parsimonious explanation for these observations appears to be that the shedding of the ZIP10 ectodomain occurs while ZIP10 traverses the secretory pathway. This timing would fit well with recent data documenting that cleavage of PrP^C at its α -site might also occur during maturation within the secretory pathway.³⁰

α -Site cleavage of PrP^C

If indeed the endoproteolysis of ZIP10 and PrP^C observed in response to manganese or zinc starvation was the work of the same endoproteolytic activity, why would this shedding cause the release of the entire ectodomain in ZIP10 *versus* the release of only the α -site N-

proximal fragment N1 from PrP^C? One angle from which to explore this question might be to compare the minimal requirements for this cleavage. Whereas previous data are missing for ZIP10 in this regard, it was recently shown that α -site cleavage of PrP^C requires the integrity of the hydrophobic domain, which is flanking this cleavage site on the C-terminal site of the scissile bond (mapped to murine residues 109/110).²⁴ More specifically, cleavage at the α -site was size dependently impaired by deletions of the hydrophobic domain spanning residues 106–119 but was surprisingly tolerant toward changes within the amino acid sequence. This description is consistent with substrate determinants of sheddases that target cleavage sites N-proximal to transmembrane domains. Thus, from the perspective of a sheddase dispatched to shed the ectodomain of LZTs in response to transition metal starvation, the cleavage of a nearby PrP^C molecule at its α -site might not seem so different. Although it has been shown that protein kinase C agonists can stimulate α -site cleavage,³¹ most reports refer to the cleavage of PrP^C at this site as the “normal” or constitutive cleavage.³⁰ The robust stimulation of PrP^C cleavage at this site in response to manganese or zinc starvation observed in this study, therefore, came as a surprise, particularly in light of a literature that seemed to have left no stone unturned in efforts to understand the transition metal biology of PrP^C. Considerable interest in the proteolytic activity responsible for α -site cleavage arises from the critical role ascribed to the hydrophobic domain for the disease-associated conversion of the prion protein. The ability to manipulate cleavages at this site therefore may provide an angle for disease intervention strategies. From the data presented in this work, it cannot currently be deduced whether the ZIP10 ectodomain cleavage observed in response to the challenge of mice with mouse-adjusted RML prions indicates that transition metal starvation exists in these mice or whether a molecular phenocopy of transition metal starvation is observed. Work by other researchers, which attempted to address whether transition metal imbalances exist in prion-infected mice, has been largely inconclusive or, at times, led to contradictory results. Recent improvements to imaging methods that now allow interrogation of the neuroanatomy of metal distributions in the brain attest to a surprising heterogeneity in the distribution of transition metals and suggest that PrP^C may indeed contribute to this biology.³²

Conclusions

Data presented in this study not only provided multiple novel angles from which to explore the biology of the prion protein in health and disease but also raised many questions that need to be answered. More work will be needed to (i) understand whether a new look at the transition metal biology of the prion protein (with emphasis on its relationship to LZTs) may provide avenues for therapeutic intervention, (ii) determine whether binding of prion proteins to LZTs contributes to differences in posttranslational signatures of the prion protein underlying well-established strain phenomena in prion disease and (iii) explore whether the LZT expression profiles of cells or the ectodomain shedding of LZTs contribute to a poorly understood biology surrounding the susceptibility of cells to prion infection or the spread of prion disease.

Materials and Methods

Cell viability assay

Mouse neuroblastoma Neuro-2a (N2a) cells were obtained from the American Type Culture Collection (cat. no. CCL-131; Manassas, VA, USA). N2a cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as reported previously.³³ Briefly, 5 mg/mL of MTT (Sigma-Aldrich, Oakville, ON, Canada) in phosphate-buffered saline (PBS) was added to the culture medium of N2a cells (+/-TPEN/DTPA) grown on 96-well plates at a final concentration of 0.25 mg/mL. After incubation for 4 h, cells were solubilized in 70% isopropanol acidified to 0.1 N HCl. The absorbances were measured at a wavelength of 550 nm. The extent of MTT conversion in N2a cells exposed to the chelating reagents was expressed as a percentage of the control.

Confocal immunofluorescence analysis

N2a cells were grown on poly-D-lysine-coated coverslips 24 h prior to and following transfection with Lipofectamine 2000 reagent (cat. no. 11668; Invitrogen, Carlsbad, CA, USA). Cells were then fixed with 4% formaldehyde in PBS for 10 min at 37 °C, and the reaction was quenched with 25 mM glycine and 25 mM ammonium chloride in PBS for 15 min at 37 °C. Following permeabilization with 0.2% Triton X-100 in PBS and blocking with 0.1% saponin, 2% bovine serum albumin and 1% horse serum in PBS, cells were incubated for 1 h with primary antibodies. After five 3-min PBS washes, fluorescently conjugated secondary antibodies were added and incubated for 1 h. Images were acquired using an inverted fluorescence microscope (DMIRE2; Leica Microsystems, Concord, ON, Canada) equipped with a back-thinned electron multiplier charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ, USA) and spinning disk confocal scan head. The unit was outfitted with four separate diode-pumped solid-state laser lines (405 nm, 491 nm, 561 nm and 638 nm; Spectral Applied Research, Richmond Hill, ON, Canada), an Improvion Piezo Focus Drive (PerkinElmer, Wood-bridge, ON, Canada), a motorized xy stage (Applied Scientific Instrumentation, Eugene, OR, USA) and a 1.5 × magnification lens (Spectral Applied Research). The equipment was controlled by Volocity acquisition software (PerkinElmer). Split Mander's co-localization co-efficients for comparing spatial signal overlaps of ZIPs and PrP were calculated in Volocity from at least 20 flattened Z-stacks (encompassing 20–30 layers) of individual cells. The analyses were conducted in three technical replicates.

Immunohistochemical staining

Five-micron-thick sections of formalin-fixed paraffin-embedded tissues of wild-type mouse brains were dewaxed in five changes of xylenes and then brought down to water through graded alcohols. Endogenous peroxidase activities were blocked for 15 min using 3% aqueous solution of hydrogen peroxide before tissues were heat-retrieved with 10 mM citrate buffer at pH 6.0. Heat-induced epitope retrieval was performed using a Decloaking Chamber (Biocare Medical, Concord, CA, USA) at 120 °C for 2 min followed by cooling off to 90 °C before slides were removed from the Decloaking Chamber.³⁴ Tissues were then allowed to stay in the hot buffer for another 20 min at room temperature before washing well in running tap water. Sections were blocked for 30 min with 2.5% normal horse serum

before incubating overnight with in-house rabbit polyclonal antibodies to ZIP10 at 1:100 dilution using an antibody diluting buffer containing Tris buffer and casein. Staining was finished using the ImmPRESS anti-rabbit Ig peroxidase kit (cat. no. MP-7401; Vector Laboratories, Burlingame, CA, USA). To validate the specificity of the stain, we generated a negative control sample by pre-saturating the polyclonal antibodies with the peptide antigen during overnight incubation. All incubations were performed at room temperature inside a moist chamber, and sections were washed thoroughly in three changes of Tris-buffered saline before color development with a freshly prepared solution of NovaRed (cat. no. SK4800; Vector Laboratories). Finally, sections were lightly counterstained with Mayer's hematoxylin (cat. no. MHS16-500ML; Sigma-Aldrich), washed well with water, dehydrated through graded alcohols, cleared in xylenes and then mounted in Permount (cat. no. SP15-500; Fisher Scientific, Nepean, ON, Canada).

Divalent metal ion measurements

An Optima 7300 spectrometer (PerkinElmer) was used for inductively coupled plasma atomic emission spectroscopy-based measurements of divalent metal ion concentrations in different cell media (ANALEST Facility, University of Toronto). The device has a typical detection in range of micrograms per liter ($\mu\text{g/L}$) to milligrams per liter (mg/L). Specifically, it has a quantifiable minimum detection of 10 ppb for zinc and 4 ppb for copper. Three reading wavelengths were selected for each element, and the spectrometer was adjusted to read for 5–10 s. A minimum of three replicate analyses were carried out to determine transition metal concentrations in selected solutions. Calibration was performed using *PlasmaCAL* Multi-Element Standard QC-4 (SCP SCIENCE, Baie d'Urfé, QC, Canada).

Glycosylation analysis

Cell lysates (20 μg of protein) were denatured in 0.5% SDS and 1% β -mercaptoethanol at 100 °C for 10 min. NP-40 and sodium phosphate were added to final concentrations of 1% and 50 mM, respectively. We incubated 5 μL (2500 units) of PNGase F (New England Biolabs, Ipswich, MA, USA) with the mixture at 37 °C for between 3 h and overnight.

Metal chelation

The cell-permeable chelator TPEN (cat. no. P4413; Sigma-Aldrich) was first dissolved in ethanol at 23.4 mM and subsequently diluted 100 $\times$ in cell culture medium to produce a 234- μM stock. The cell-impermeable chelator DTPA (cat. no. D6518; Sigma-Aldrich) was dissolved in cell culture medium for a 1.2-mM stock solution. Both solutions were filter sterilized before addition to cells and incubated for 48 h, unless indicated otherwise.

Molecular cloning

ZIP10 (Slc39a10 open reading frame in pCMV6-Entry) was obtained from OriGene (Rockville, MD, USA). A C-terminal HA-tag (YPYDVPDYA) was introduced in the plasmid following a glycine spacer residue. ZIP10-HA_d300 (deleted from residue 31 to residue 300), ZIP10-HA_d389 (deleted from residue 36 to residue 389), ZIP10-HA_N341A and ZIP10-HA-(H711A/H715A) were derived from the ZIP10-HA plasmid. The HotStar

HiFidelity polymerase (Qiagen, Valencia, CA, USA) was used for site-directed mutagenesis. All plasmids were sequence verified.

Polyclonal antibody generation

A KLH-conjugated peptide derived from mouse ZIP10 (CNHHDHSEQYEHNR) was synthesized and injected into Charles River Laboratories SPF rabbits (Division of Comparative Medicine, University of Toronto). Upon exsanguination, a portion of serum was purified using the SulfoLink Immobilization Kit for Peptides (cat. no. 44999; Pierce, Rockford, IL, USA) following instructions provided by the manufacturer.

Reverse transcription PCR

cDNAs were generated from the total RNA (5 µg) of N2a cells or mouse brain (CD-1) using the StrataScript first-strand synthesis kit (Stratagene, La Jolla, CA, USA). PCR primers were designed using Primer3 software (S. Rozen and H. Skaletsky, Whitehead Institute for Biomedical Research) as follows:

ZIP4: F, AGACTTGGTGGCAGAGGAGA;

R, GGGATGGGATAGAGCTGTCA

ZIP5: F, CTCCCCTCTCTCTGCTGTTG;

R, AGAAGCCGTCTGAGAAAGCA

ZIP6: F, GGATGTGGAGAGCAAGAAGC;

R, CACAGCGACAGAGGTGCTTA

ZIP8: F, AGCAGTTGCTGTGTTTGGTG;

R, GCGGAGAGGAAGTTGAACAG

ZIP10: F, TTACCTGGGTGTGGAGGAAG;

R, TTGCGAAGATCCAGAGTGTG

ZIP14: F, TGCCTCCTTCACTGTGTCTG;

R, AAGAGCTGCCTTTTCCATGA

A reaction volume of 50 µL containing 2 µL cDNA, 25 pmol of each primer, 5 µL PCR reaction buffer (Qiagen) and 2.5 U Taq polymerase (Qiagen) was thermocycled for 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The resulting PCR fragments were resolved on a 1.5% agarose gel.

Preparation of mouse brain homogenates

Scrapie inoculations with mouse-adapted RML prions were carried out as previously described.^{35,36} Briefly, FVB/NCr *prnp*^{+/+} mice were intracerebrally inoculated in the left hemisphere with 20 µL of 1:10 (v/v) diluted brain homogenate in PBS, sourced from either clinically ill RML-infected or normal mice. Following 84 or 131 (or 143) days p.i., the right hemispheres of mice were harvested and sliced into small sections. To homogenize the brain pieces, we added a 10-fold excess (v/w) of Extraction Buffer [Tris-HCl (pH 7.5), 2% SDS

and 100 mM DTT], followed by a 5-min incubation at 90 °C and three consecutive 1-min bead beating steps at “homogenization” speed, with the steps interrupted by 1-min breaks (Mini Beadbeater-8; BioSpec Products, Bartlesville, OK, USA) in the presence of 1-mm zirconia beads (BioSpec Products). Samples were spun for 20 min at 38,000g to remove insoluble debris and, subsequently, diluted in concentrated Laemmli sample buffer to adjust to a final concentration of 2% SDS, 100 mM DTT, 30% glycerol, 60 mM Tris-HCl (pH 6.5) and 0.001% bromophenol blue.

SDS-PAGE and immunoblotting

For denaturing SDS-PAGE analyses, protein mixtures were separated at 180 V in 4–12% or 12% NuPAGE 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol gels (Invitrogen) in Mops running buffer. The transfer of proteins to polyvinyl fluoride membranes was accomplished within 1.5 h at 40 V. Subsequently, membranes were probed overnight with one of the following primary antibodies: anti-Drebrin (1:100, cat. no. D029-3; MBL, Woburn, MA, USA), anti-Munc18 (1:10,000, cat. no. 610336; BD Transduction, Mississauga, ON, Canada), anti-MnSOD (1:1000, cat. no. SOD-110D; Enzo Life Sciences, Farmingdale, NY, USA), anti-Histone H4 (1:1000, cat. no. 04-118; Millipore, Billerica, MA, USA), anti-TfR (1:500, cat. no. 13-6800; Invitrogen), anti-CCS (1:200, cat. no. sc-20141; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH (1:2000, cat. no. G8795; Sigma-Aldrich), anti-ZIP10 (1:800), anti-HA.11 (1:1000, cat. no. MMS-101P; Covance, Princeton, NJ, USA), anti-PrP rabbit monoclonal (1:5000, cat. no. 2063-1; Epitomics, Burlingame, CA, USA) or anti-PrP Sha31 (1:5000, cat. no. A03213; Mediacorp, Montreal, QC, Canada). Membranes were then incubated with anti-mouse (1:10,000, cat. no. 170-6516; Bio-Rad, Mississauga, ON, Canada) or anti-rabbit (1:10,000, cat. no. 170-6515; Bio-Rad) secondary antibody and visualized using ECL reagent (cat. no. RPN2106; GE Healthcare, Baie d'Urfe, QC, Canada). For band quantification, the histogram feature of Photoshop CS5 (Adobe Systems, San Jose, CA, USA) was used, and two-sample, two-tailed Student's *t*-test assuming unequal variance was utilized for tests of statistical significance.

Proteinase K digestion

Brain extracts were digested with 12 µg/mL of proteinase K for 30 min at 37 °C. The reaction was stopped with 2 mM PMSF and incubated with 0.7% phosphotungstic acid and 0.1% sodium lauroyl sarcosinate for 30 min at 37 °C to precipitate PrP^{Sc}. The samples were subsequently pelleted, resuspended in sample buffer and heated at 90 °C for 10 min.

Sucrose gradient isolation of lipid rafts

Lipid rafts (also referred to as *caveolae*-like membrane domains) were isolated as described previously.^{37,38} Briefly, confluent cells were washed twice in ice-cold PBS (pH 7.2) and lysed in a lysis buffer containing Triton X-100 [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 1 × Roche Complete Protease Inhibitor Cocktail]. After 20 min of incubation on ice, cells were scraped and homogenized through successively higher-gauge syringe needles. The lysate was diluted 1:1 in 80% sucrose and placed at the bottom of a 38.5-mL open-top thin-wall ultra-clear ultracentrifuge tube (Beckman Coulter, Indianapolis, IN, USA). Two stepwise sucrose layers of (i) 25% and (ii) 5% were poured (at a rate of < 0.5 mL/min) above the lysate. All sucrose dilutions were prepared in lysis buffer lacking Triton

X-100. Ultracentrifugation was performed for 21 h at 28,000 rpm (140,000*g*) in a Beckman Coulter Optima L-100 XP ultracentrifuge and SW 32 Ti rotor. Upon completion of centrifugation, the 5%, 5–25% transition (representing the lipid raft fraction), 25% and lysate layers were collected. The 5–25% transition layer was diluted with lysis buffer, pelleted and subsequently resuspended in 1 × Laemmli sample buffer. All other layers were diluted 1:1 with 2 × sample buffer. Lastly, the ultracentrifugation pellet was dissolved in 1 × Laemmli sample buffer directly in the ultracentrifuge tube. All sample-buffer-containing samples were analyzed using Western blotting.

Transient overexpression and knockdown

Mouse neuroblastoma (N2a) cells were cultured in minimum essential medium (cat. no. 11095; Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (cat. no. 12484028; Gibco), 1% GlutaMAX (cat. no. 35050; Gibco) and 1% antibiotic/antimycotic solution (cat. no. 15250-062; Gibco). Lipofectamine 2000 transfection reagent (Invitrogen) was used as per manufacturer's instructions. For transient overexpressions, 5 mM sodium butyrate (cat. no. B5887; Sigma-Aldrich) was added to cells 24 h post-transfection from a 1.5-M stock solution in water. The transfection duration was 48 h. Cells were lysed in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholate and 1 × Roche Complete Protease Inhibitor Cocktail], followed by bicinchoninic acid protein adjustment and 10-min heating at 45 °C in Laemmli sample buffer before SDS-PAGE analysis.

Supplementary Data

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CFC	cysteine-flanked core
DTPA	diethylenetriamine pentaacetic acid
HA	hemagglutinin
i.c.	intracerebral
LZT	LIV-1 subfamily of ZIP zinc transporters
MW	molecular weight
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
N2a	Neuro-2a

PBS	phosphate-buffered saline
p.i.	post-inoculation
PrP	prion protein
RML	Rocky Mountain Laboratory
TPEN	<i>N,N,N',N'</i> -tetrakis(2-pyridylmethyl)ethylenediamine

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Fig. 1.

Prion disease in mice causes appearance of a novel protein band that can be detected with ZIP10-directed antibody. Mouse littermates were inoculated with 10% brain homogenates generated from the brains of naïve control mice or mice that had been infected with mouse-adapted RML prions. At 84 or 131 days following i.c. challenge with prions, the mice were sacrificed, and their brain homogenates were adjusted for equal protein concentration and side-by-side analyzed by Western blotting with antibodies directed against PrP^C or ZIP10. Please note the appearance of a protein band that is barely visible in control mice but can be detected with the ZIP10-directed antibody in RML-inoculated mice (marked with an arrowhead). PrP^{Sc} infection in the 131d RML brain was confirmed by the presence of high-MW aggregates and relative proteinase K resistance. Based on the known epitope specificity of the anti-ZIP10 antibody (Fig. 2a), the novel band can be inferred to comprise the respective PrP-like ectodomain of ZIP10. The apparent MWs of additional signals detected with the anti-ZIP10 antibody are indicated. Control Western blots with antibodies that can probe for GAPDH or cellular transporters of iron (TfR) and copper (CCS) revealed no differences in the signal levels of these proteins in brain homogenates derived from negative control or prion-challenged mice. Please see Fig. S1 for quantitative analyses of levels of additional cell status reporters.

Fig. 2.

ZIP10 is localized at the plasma membrane and undergoes complex posttranslational modifications. Cellular distribution and posttranslational processing of ZIP10 in N2a cells. (a) Schematic diagram depicting targeted epitopes within ZIP10 and amino acid sequence of synthetic peptide antigen used for conjugation to KLH and for raising polyclonal rabbit antibodies. (b) N2a cells were transfected with a ZIP10-HA expression vector and the distribution of the heterologously expressed protein analyzed by immunofluorescence. A comparison of bright field, extended focus and confocal views revealed predominant

localization of ZIP10 at the plasma membrane and its association with punctate structures seen within the cell and within neuritic membrane protrusions (see inset). Please note that a negligible background stain was observed in non-transfected cells (data not shown). (c) Immunohistochemical analysis of endogenous ZIP10 on formaldehyde cross-linked and paraffin-embedded mouse brain sections revealed predominant punctate staining of plasma membranes of cortical neurons. Immunohistochemical staining generated with ZIP10-directed polyclonal antibodies that had been pre-saturated with their cognate peptide served as negative controls. (d) Transient transfection of ZIP10-HA followed by detection with anti-HA antibody recognizing C-terminal HA-tag or with anti-ZIP10_N antibody. A comparison of signals revealed the SDS-stable ZIP10 dimer to probably represent an overexpression artifact and identified two C-terminal cleavage products that were designated as C1 and C_ecto on the basis of evidence revealed in experiments described below. The asterisk indicates an unspecific band. (e) Cellular extracts of ZIP10-HA-transfected cells digested with PNGase F were analyzed next to undigested extracts. A comparison of band patterns indicated that both full-length ZIP10 and the C-terminal fragment C1, but not C_ecto, were N-glycosylated.

Fig. 3.

ZIP10 is N-glycosylated at “NxT” acceptor site it shares with PrP^C and is partially shed from the membrane by at least two distinct cleavages. (a) Schematic presentation of ZIP10-HA-derived expression constructs used in subsequent experiments. Colors and symbols are explained in the legend to the right of the schematic. (b) Transient expression analysis of ZIP10 expression constructs introduced in (a) with and without prior digestion of extracts with PNGase F. A comparison of bands revealed the N341 residue within the “NxT” acceptor site to be quantitatively N-glycosylated. Please note, however, that additional N-glycosylation sites must exist since digestion with PNGase F leads to a 3- to 4-fold larger shift in the apparent MW of the slow-migrating full-length ZIP10 signal. Please also note the similarity in apparent MWs of C-terminal cleavage products C1 and C_ecto to the length of ZIP10 expression constructs with N-terminal truncations that extend to amino acids 300 and 389, respectively. Extract amounts analyzed were adjusted for total protein levels.

Fig. 4.

Depletion of zinc and manganese but not copper from the cell culture medium causes shedding of the ZIP10 ectodomain and cleavage of PrP^C in N2a cells. N2a or GT1 cells were grown for 2 days in cell culture media that were either supplemented with defined concentrations of individual transition metals or functionally depleted of transition metals by the addition of chelation agents. Subsequently, the posttranslational processing of PrP^C and ZIP10 was characterized by immunoblotting of cellular extracts. (a) Whereas both ZIP10 and PrP^C demonstrate only subtle changes in their overall full-length expression levels in the

presence of an excess of transition metals, both proteins undergo endoproteolysis when transition metals were depleted by the cell-permeable transition metal chelator TPEN. (b) To determine the cellular tolerance toward the use of transition metal chelators in the cell culture medium, we conducted an MTT assay analysis with a range of TPEN or DTPA concentrations. (c) DTPA-induced ZIP10 cleavage causes the majority of N-terminal ZIP10 fragments to be shed into the cell culture medium. On the basis of calculations considering the relative amounts of cell culture volume and cell pellet analyzed, the ratio of shed *versus* cell-bound ZIP10 fragments can be estimated to exceed 95%. (d) A phenotypic rescue experiment was conducted to determine the identity of the transition metal(s) that can rescue the DTPA-mediated endoproteolysis of PrP and ZIP10. The experimental steps were as for (a), except that individual transition metals were added to the cell culture medium concomitantly with DTPA at the concentrations indicated and that the data are presented in composite Western blots. (e) No endoproteolysis of PrP or ZIP10 was observed when analogous experiments were conducted with GT1 cells. Of note, however, are the opposite effects of DTPA or DTPA plus 50 μ M zinc on full-length PrP^C and ZIP10 protein levels that echoed observations made in N2a cells. Note that the absence of the cleavage products may reflect that endoproteolytic activities are less pronounced in these cells or that proteolytic fragments are more effectively cleared.

Fig. 5.

A conserved zinc coordination site within TMD5 influences N-glycosylation and shedding of the ZIP10 ectodomain. Altered processing of ZIP10, in which histidines within a putative metalloproteinase catalytic center characterized by the "CHELPHELGDF" motif were mutated to alanines. (a) Multiple sequence alignment of mouse ZIP paralog sequences in the region surrounding a putative zinc metalloproteinase catalytic center present within the predicted TMD5 in a majority of sequences belonging to the LZT branch of ZIP transporters. (b) N2a cells were transiently transfected with wild-type ZIP10-HA or a mutant ZIP10-HA-(H711A/

H715A), and following 48 h of cell growth, cell extracts were analyzed by immunoblotting. Please note the differences in the apparent MWs of a subset of ZIP10 wild-type and ZIP10 mutant expression products. The numbering 1 to 4 shown next to the N_{ecto} label indicates the number of *N*-glycans present within individual ZIP10 ectodomains observed [see also (c)–(e) for evidence in support of this interpretation]. (c) When cells expressing mutant ZIP10-HA were grown in the presence of DTPA, the generation of the ZIP10 N_{ecto} cleavage product modified by a single *N*-glycan takes precedence over the generation of more fully *N*-glycosylated N_{ecto} cleavage products. Please note that the MW ladder indicates protein weights in both Western blots. (d) Cellular extracts obtained from naïve N2a cells or N2a cells grown in the presence of DTPA were subjected to digestion with PNGase F at a low (+) or high (++) enzymatic dose. All ZIP10 cleavage products merged into a single band when fully digested with PNGase F. (e) Similarly, cleavage products of heterologously expressed ZIP10-HA-(H711A/H715A) all converted to a band of the same apparent MW when digested with a high dose of PNGase F.

Fig. 6.

The novel ZIP10-reactive band observed in prion-infected mice phenocopies ZIP10 ectodomain shedding in response to zinc or manganese depletion and is distinct from constitutive ZIP10 ectodomain shedding. A comparison of PrP and ZIP10 cleavage products observed upon chelation of transition metals from the medium *versus* RML inoculation is depicted. (a) Both transition metal chelation and prion infection led to PrP^C cleavage products that could be detected with a PrP-directed antibody (Sha31) that binds to the respective C-terminal PrP^C stubs retained on the plasma membrane. Upon PNGase F

digestion, these PrP^C cleavage products were revealed to be distinct. Whereas the PrP^C C1 fragment appeared to have been accumulated in transition metal-depleted cells (identified by its characteristic deglycosylated apparent MW; please note that a small amount of partially deglycosylated C1 migrating slightly above can also be seen), the PrP^C C2 fragment accumulated in prion-infected cells or brain (depicted in composite Western blots). (b) In contrast, transition metal depletion or prion infection caused the appearance of a ZIP10 endoproteolytic cleavage product of the same apparent MW. On the basis of data in Fig. 5, this cleavage product was identified as the ZIP10 N_{ecto} cleavage product carrying one *N*-glycan. (c) Schematic drawing summarizing epitopes of antibody binding sites relative to endoproteolytic cleavages and *N*-glycosylation sites observed in this study. (d) Cartoon summarizing a mechanistic model consistent with data presented herein. According to this model, (i) wild-type ZIP10 undergoes complex maturation processes that lead to the attachment of up to four *N*-glycans to its ectodomain but not the channel domain. The ectodomain may make contact with the conserved CHELPHEL sequence embedded in the putative TMD5 of LIV-1 ZIP transporters. (ii) Disruption of this ectodomain–TMD5 interface by the introduction of point mutations that eliminate a transition metal coordination site or by depletion of Mn²⁺ or Zn²⁺ from the extracellular milieu causes shedding of the ZIP10 ectodomain (N_{ecto}). (iii) Whereas this shedding event follows ZIP10 maturation (as evidenced by the presence of two to four *N*-glycans on the N_{ecto} cleavage product) in cells expressing the ZIP10-(H711A/H715A) mutant, endoproteolysis at the same (or a very nearby site) occurs on immature ZIP10 (as evidenced by the presence of only a single *N*-glycan) in Mn²⁺- or Zn²⁺-depleted medium or in mouse brains inoculated with RML prions.