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Metabolism of minor isoforms of prion proteins

Cytosolic prion protein and transmembrane prion protein

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Research Highlights

Two types of disease-associated prion proteins, the cytosolic form and the transmembrane form, are introduced during biosynthesis through the endoplasmic reticulum quality-control system. Here, we propose an effective model and testing method for cytosolic forms of prion protein. Studies addressing the molecular mechanisms show that the pathogenic factor for prion disease is not limited to the prion protein, but also includes the complex intracellular environment, error location or incomplete removal of topologic prion protein.

Abstract

Transmissible spongiform encephalopathy or prion disease is triggered by the conversion from cellular prion protein to pathogenic prion protein. Growing evidence has concentrated on prion protein configuration changes and their correlation with prion disease transmissibility and pathogenicity. *In vivo* and *in vitro* studies have shown that several cytosolic forms of prion protein with specific topological structure can destroy intracellular stability and contribute to prion protein pathogenicity. In this study, the latest molecular chaperone system associated with endoplasmic reticulum-associated protein degradation, the endoplasmic reticulum resident protein quality-control system and the ubiquitination proteasome system, is outlined. The molecular chaperone system directly correlates with the prion protein degradation pathway. Understanding the molecular mechanisms will help provide a fascinating avenue for further investigations on prion disease treatment and prion protein-induced neurodegenerative diseases.

Key Words

neural regeneration; neurodegeneration; prion protein; cytosolic form of prion protein; transmembrane form of prion protein; metabolism; protein degeneration; ubiquitination; molecular chaperone; molecular mechanism; neuroregeneration

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INTRODUCTION

The mammalian prion protein is a cell-surface glycosyl phosphatidyl inositol-link glycoprotein implicated in several neurodegenerative diseases. The diseases include kuru, Creutzfeldt-Jakob disease[1], Gerstmann- Sträussler-Scheinker disease, and fatal familial insomnia in humans, natural scrapie in sheep, goats, and mouflons, transmissible mink encephalopathy in ranch-reared mink, chronic wasting disease of mule deer and elk in North America, and bovine spongiform encephalopathy or "mad cow disease"[2-3]. They are fatal brain diseases of animals and humans and belong to the group of protein misfolding neurodegenerative diseases.

Transmissible spongiform encephalopathy and prion diseases are synonymous: both refer to the spectrum of slowly progressing, transmissible neurodegenerative diseases with characteristic clinical and pathological sequelae[4-6]. The term prion, derived from the words proteinaceous and infectious, is by definition the transmissible agent in these diseases[4]. Thus, a prion is an agent responsible for the transmission of prion diseases (or transmissible spongiform encephalopathy) from one organism to another. No treatment is available to halt the rapidly progressing neurodegenerative disease once prions have reached the brain. The scrapie-associated prion protein, an abnormally structured and aggregated form of the host prion protein, is thought to be the only[7-10] or the major[11] constituent of the infectious agent called prion. However, this view appears to be overly simplistic because several experimental paradigms have partially or fully uncoupled prion protein aggregate deposition from downstream neuropathology[12-15]. Conversely, several familial prion protein mutations cause neurodegeneration with little or no generation of scrapie-associated prion protein, or transmissible agent[14, 16], prompting investigation into other isoforms of prion proteins that may mediate neurotoxicity.

The general term prion protein is used to refer to this protein when no single form is being specified, *e.g.*, in prion protein-mediated neurodegeneration. However, with the development of science and technology, this single protein has been described in numerous forms and locations. Prion protein is often appended with a modifier to more specifically refer to one particular species among several that include: cellular prion protein, scrapie-associated prion protein, protease-resistant prion protein, protease-sensitive prion protein, secretory prion protein, C-transmembrane form of prion protein, cytosolic prion protein, and N-transmembrane form of prion protein (Figure 1).

Protease-resistant prion protein and protease-sensitive prion protein define species of prion protein on the basis of their biochemical property of protease resistance. The remaining forms of prion protein (the C-transmembrane form of prion protein, cytosolic prion protein, N-transmembrane form of prion protein, and secretory prion protein) make distinctions based on the cellular locale and topological orientation of prion protein relative to a membrane. Sequence analysis of the full-length prion protein open reading frame suggested an N-terminal signal for targeting to the endoplasmic reticulum and two potential sites for N-linked glycosylation in the C-terminal domain. In addition, the sequence revealed a hydrophobic domain of about 20 residues and a downstream amphipathic region. These elements were incorporated together into a model of prion protein as a double-spanning transmembrane protein in which the N- and C-termini were in the lumen. When certain kinds of prion proteins fail to translocate into the endoplasmic reticulum, they generate a minor cytosolic form of prion protein. After targeting to the translocon, the signal sequence fails to initiate translocation of the N terminus, an internal hydrophobic domain at residues 112–135, which can engage the translocon and direct membrane insertion of the prion protein. This transmembrane form, termed the C-transmembrane form of prion protein, has the N-terminus in the cytosol and C-

terminus in the endoplasmic reticulum lumen.

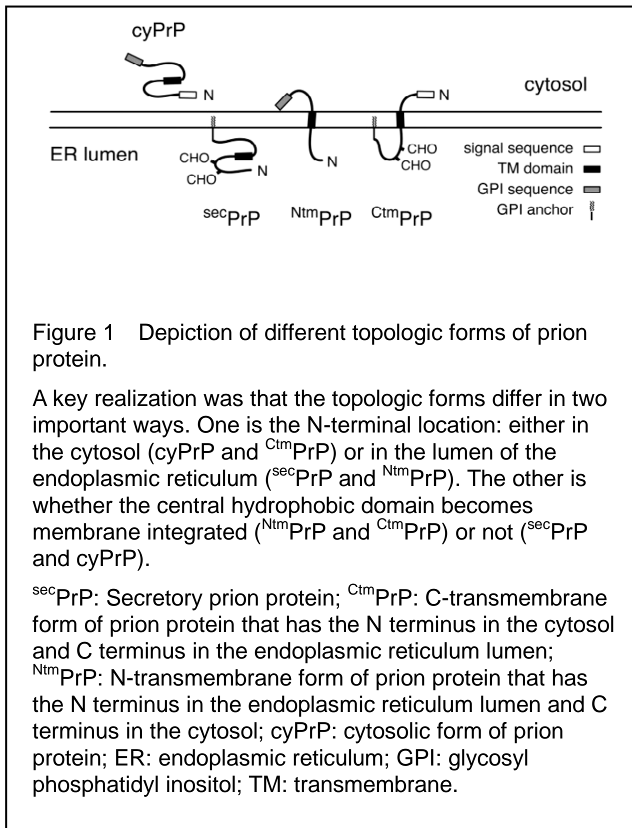

Figure 1 Depiction of different topologic forms of prion protein.

A key realization was that the topologic forms differ in two important ways. One is the N-terminal location: either in the cytosol (cyPrP and ^{Ctm}PrP) or in the lumen of the endoplasmic reticulum (^{sec}PrP and ^{Ntm}PrP). The other is whether the central hydrophobic domain becomes membrane integrated (^{Ntm}PrP and ^{Ctm}PrP) or not (^{sec}PrP and cyPrP).

^{sec}PrP: Secretory prion protein; ^{Ctm}PrP: C-transmembrane form of prion protein that has the N terminus in the cytosol and C terminus in the endoplasmic reticulum lumen; ^{Ntm}PrP: N-transmembrane form of prion protein that has the N terminus in the endoplasmic reticulum lumen and C terminus in the cytosol; cyPrP: cytosolic form of prion protein; ER: endoplasmic reticulum; GPI: glycosyl phosphatidyl inositol; TM: transmembrane.

The N-transmembrane form of the prion protein was in exactly the reverse orientation, *i.e.*, it spanned the membrane with the N-terminus in the endoplasmic reticulum lumen and the C-terminus in the cytosol.

First, cytosolic prion protein has altered susceptibility to aggregation[17], suggesting that these neurons might play a significant role in the pathogenesis of prion diseases, in particular those mammals harboring mutant prion protein genes.

Second, recently published studies addressing the effects of proteosome inhibitors on cellular prion protein degradation and expression of cytosolic prion protein have suggested that cytosolic localization of prion proteins is sufficient to induce neurodegeneration. The disease-associated prion protein mutation (D178N) was found to exhibit higher expression in the cytosol than wild type prion protein under both normal and proteasome-inhibited conditions. However, it remains to be explored whether cytosolic prion protein aggregates are in fact transmissible and propagated when introduced into animals.

Third, the minor isoform of prion protein, termed the C-transmembrane form of prion protein, spans the membrane once at a hydrophobic domain from residues 112–135 with the N-terminal domain exposed to the cytosol[18], and is the transmembrane isoform associated with disease. Remarkably, both the natural and several artificial mutants within the hydrophobic domain have been shown to lead to a modest increase in the generation of the C-transmembrane form of prion protein (5–20% of total prion protein) and cause neurodegeneration in transgenic mice[19]. Interestingly, there was a dose-response effect. When greater amounts of the C-transmembrane form of prion protein were present, the development of neurodegeneration lacking scrapie-associated prion protein was earlier. Conversely, ΔSTE (delta stop transfer effector) and G123P mutations that reduce or abolish the ability to generate either of the transmembrane forms did not lead to neurodegeneration. In extensive attempts at transmission involving hundreds of recipients, the C-transmembrane form of prion protein-mediated neurodegeneration was shown to be non-transmissible. Thus, with the accumulated data from four different C-transmembrane forms of prion protein-favoring mutations and two transmembrane-disfavoring mutations, each in multiple lines of transgenic mice at different expression levels, a very strong positive correlation can be made between the ability to generate the C-transmembrane form of prion protein and neurodegenerative disease[18].

CORRELATION OF THE C-TRANSMEMBRANE FORM OF PRION PROTEIN AND CYTOSOLIC PRION PROTEIN WITH NEURODEGENERATIVE DISEASES

When the gene encoding prion protein was first cloned, an obvious and deceptively simple question was to determine its normal biosynthetic pathway and cellular locale. With a model supported following the initial analysis of prion protein topology upon its *in vitro* synthesis using wheat germ extracts and endoplasmic reticulum microsomes derived from canine pancreas, the view of prion protein as a transmembrane protein was short lived. In addition, in cells, the protein was found to be fully exposed on the extracellular surface, where it was discovered to be tethered to the plasma membrane by a C-terminal glycolipid anchor. Thus, original topology predictions and results from wheat germ translation systems were largely ignored, presumed to be an artifact of using a plant-based system to analyze a mammalian protein. Given the long-standing and widely held belief that each protein has a single ‘correct’ final configuration, it was concluded that normal cellular prion protein is a

glycosyl phosphatidyl inositol-anchored cell surface glycoprotein. All other observed forms were thought to represent either mistakes or artifacts, and hence, irrelevant to normal prion protein function. This is the view that generally persists today. Curiously however, the transmembrane form of prion protein, exaggerated in the wheat germ system (> 80% of total prion protein, depending on translation conditions), is nonetheless also observed (at an albeit lower level of 5–10%) in the reticulocyte lysate system[18]. This topological heterogeneity was not observed for any of the numerous secretory models or membrane proteins that had been examined in *in vitro* translocation systems. Furthermore, the central hydrophobic domain of prion protein that allows it to potentially span the membrane was subsequently found to be extremely well conserved across species. This highly conserved, albeit unusual feature that is required for a proportion of the prion protein to be made as a membrane-spanning protein suggested an alternative explanation for the transmembrane form.

Unfortunately, lack of a clear functional role for prion protein made this hypothesis difficult to explore. A related idea that transmembrane prion protein could play a role in disease was not considered because more dramatic observations of scrapie-associated prion protein accumulation suggested a more obvious culprit.

However, several concurrent studies demonstrate that scrapie-associated prion protein formation was clearly associated with disease transmission, and its accumulation was not inherently toxic to neurons. The first hint was when mice heterozygous for the prion protein gene (prion protein$^{+/-}$) were inoculated with prions. Scrapie-associated prion protein accumulation was very similar to that in wild type mice (*i.e.*, prion protein$^{+/+}$). The progression to clinical neurodegenerative disease was markedly delayed[20]. This discordance between scrapie-associated prion protein and neuronal damage was particularly dramatic in brain grafting experiments, where prion protein knockout neurons appeared impervious to any adverse consequences of scrapie-associated prion protein deposition. In parallel, the identification of the prion protein gene made it possible to discover a wide range of inherited prion protein mutations that led to familial forms of prion protein-mediated disease[21]. Biochemical analyses of tissue from such familial cases suggested that while some of them had accumulated scrapie-associated prion protein, others were surprisingly devoid[22-25]. Such biochemical results were, over the course of several years, corroborated by extensive transmission studies[26-27]. By the middle 1990s, it was reasonable to consider that prion protein-mediated neurodegeneration could be caused by non-scrapie-associated prion protein forms, other than through scrapie-associated prion protein accumulation.

It is ironic and perhaps meaningful that as the transmembrane prion protein was initially discovered as a likely ‘artifact’ of expression in a heterologous wheat germ system, cytosolic prion protein was also first noticed when expression of mammalian prion protein was attempted in the yeast system. In yeast cells, prion protein appears to be very inefficiently translocated into the endoplasmic reticulum, even when a native signal sequence from the yeast Kar2 protein is used[28]. What led to further investigation was the finding that the non-glycosylated, non-disulfide bonded, cytosolic prion protein was prone to aggregation, was insoluble, and partially resistant to protease digestion[28-29]. Although proteins in the wrong cellular compartment of a non-native organism are often misfolded, the superficial resemblance between prion protein aggregates in the yeast cytosol and scrapie-associated prion protein in mammalian prion disease provided the basis for an interesting hypothesis[28-29]: perhaps even in mammalian cells, prion protein in the cytosol could be the origin for initial generation of scrapie-associated prion protein. This hypothesis then raised the important questions of whether in mammalian cells, prion protein or disease-associated mutants can ever reside in the cytosol, and if so, what relevance this would have for either scrapie-associated prion protein formation or disease pathogenesis. The issue of whether prion protein can potentially reside in the cytosol was initially addressed indirectly by demonstrating that in cultured cells overexpressing prion protein, a small proportion of it was degraded by a pathway that could be inhibited by proteasome inhibitors[29-31]. Thus, upon treatment of cells with such inhibitors, an unglycosylated, presumably cytosolic form of prion protein accumulated. This form was found to be aggregation prone, insoluble in mild detergents, and at least partially resistant to protease digestion. Hence, under the appropriate conditions (overexpression and chronic proteasome inhibition), mammalian prion protein could reside in the cytosol of mammalian cells.

BIOSYNTHESIS OF THE CYTOSOLIC PRION PROTEIN AND C-TRANSMEMBRANE FORM OF PRION PROTEIN AT THE CELLULAR LEVEL

Because cellular prion protein and scrapie-associated

prion protein were known to be glycosylated, prion protein was presumed to be trafficked through the secretory pathway. Indeed, the biogenesis of cellular prion protein is characterized by a series of cotranslational and post-translational modifications[32]. It involves import of the nascent chain into the endoplasmic reticulum and the attachment of two N-linked core glycans and a glycosyl phosphatidyl inositol anchor. After processing of the glycans into complex structures in the Golgi compartment, cellular prion protein was targeted to the outer leaflet of the plasma membrane.

But cellular prion protein targeting and translocation to the endoplasmic reticulum[33] as well as being delivered to their final destinations are not perfectly efficient.

The dislocation of prion protein from the endoplasmic reticulum to the cytosol has been demonstrated previously in cell culture systems under certain conditions, such as in a reducing environment and glycosylation or proteosomal inhibition[30]. This could explain the presence of cytosolic prion protein *in situ*. It is plausible that the combined effects of a weak prion protein signal sequence[34-35] reduces prion protein translocation during endoplasmic reticulum stress[36-37], which decreases proteasome activity upon scrapie-associated prion protein accumulation[38-39], and disrupts the shuttling factor system. This retro translocation intermediate can accumulate on the endoplasmic reticulum membrane and accompany it to the proteasome[40-41], which may result in cytosolic prion protein generation in sufficient amounts during prion disease and contribute to neurodegeneration. At the same time, transfected cytosolic prion protein appears to be not only toxic in both cell culture and transgenic animals[37], but also in a cell type-dependent manner.

On the other hand, prion protein as a transmembrane protein is very short lived. When synthesized in a mammalian translation system (rabbit reticulocyte lysate) with pancreatic endoplasmic reticulum microsomes, prion protein fully translocated across the membrane[42]. When expressed in transgenic mice on a prion protein-null background, prion protein mutants (10–50% of C-transmembrane form of prion protein generation, in relation to about 5% for wild-type) that favor transmembrane forms of prion protein may trigger the development of neurodegenerative disease[18]. Furthermore, to study the C-transmembrane form of the prion protein, scientists used a selective labeling strategy in which spatially restricted expression of a biotinylating enzyme was combined with asymmetric engineering of the cognate acceptor sequence into the prion protein[43]. When this Biotagged prion protein is expressed in cells containing BirA in the cytosol, only the C-transmembrane form of the prion protein should be both biotinylated and glycosylated. The majority of the prion protein would be fully translocated into the endoplasmic reticulum lumen co-translationally, thereby escaping biotinylation (but not glycosylation). By contrast, the cytosolic prion protein would be biotinylated but not glycosylated, and the N-transmembrane form of the prion protein would be neither glycosylated nor biotinylated. Using this method, even wild-type prion protein generates small amounts of the C-transmembrane form of the prion protein.

As shown in Figure 2, we demonstrate the key steps during prion protein biogenesis at the endoplasmic reticulum. The results summarized in the figure reveals several important points. First, the key decisions that influence the outcome of prion protein biogenesis (with respect to topology) are made during the synthesis of prion protein (*i.e.*, cotranslationally). Second, each step is influenced substantially by interactions between the translocon and elements in prion protein (the signal sequence and transmembrane domain). Third, these interactions appear to occur with only moderate fidelity, a feature that is critical to the generation of topologic heterogeneity. Fourth, the strength of these interactions can be changed by mutations in the signal or transmembrane domain to influence the outcome of prion protein topogenesis in predictable ways. These insights not only provide a framework for understanding prion protein topogenesis, but facilitate subsequent studies on the most important mechanistic steps related to disease pathogenesis.

CRITICAL ACCESSORY COMPONENTS PARTICIPATE IN PRION PROTEIN BIOGENESIS AT THE ENDOPLASMIC RETICULUM

The C-transmembrane form of prion protein and cytosolic prion protein are inserted into the endoplasmic reticulum by two highly conserved parallel pathways. The well-studied co-translational pathway uses signal recognition particles[46-47] and its receptor for targeting and the Sec61 complex[48-49] translocon for membrane integration. A recently discovered post-translational pathway uses an entirely different set of factors involving transmembrane domain-selective cytosolic chaperones and an accompanying receptor at the endoplasmic reticulum[50].

In the first pathway, the critical step in the biosynthesis process is the signal sequence-mediated translocation of

the N-terminus into the endoplasmic reticulum lumen. The degree of inefficiency at this step determines the percent of nascent prion protein chains that have the opportunity to be made into the C-transmembrane form of prion protein and/or cytosolic prion protein.

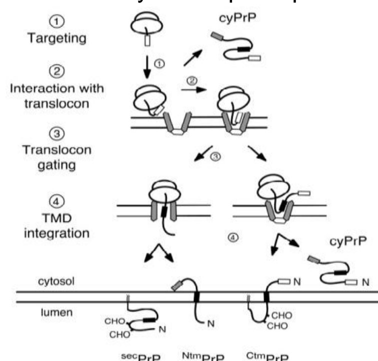

Figure 2 A mechanistic depiction of the key steps during prion protein biogenesis at the ER.

① Targeting of ribosome-associated PrP nascent chains to the ER.

This step requires a functional signal sequence, occurs by the time 50–70 amino acids are synthesized, and presumably involves the well-characterized signal recognition particle and signal recognition particle-receptor pathway[44]. Targeting to the ER appears to be essential for the generation of ^{Ctm}PrP, ^{Ntm}PrP, and ^{sec}PrP; in the absence of a functional signal sequence, PrP is made exclusively in the cytosol[45].

②–③ Before the TMD is synthesized and emerges from the ribosome, there is a brief window of time when the signal sequence mediates the insertion of nascent PrP into the ER translocation channel.

This facilitates the subsequent translocation of the N-terminus into the ER lumen, a prerequisite for the generation of ^{sec}PrP and ^{Ntm}PrP. For nascent polypeptides that fail to accomplish this step in a timely manner, the N-terminus remains on the cytosolic side of the membrane, although ribosome-nascent chain complex remains in close proximity to the translocon while the cyPrP is synthesized.

④ TMD is synthesized and PrP emerges from the ribosome.

If the N-terminus has already been committed to the ER lumen, determinants in the TMD (primarily hydrophobicity) influence the chain propensity to become membrane integrated (to generate ^{Ntm}PrP) or fully translocated (to become ^{sec}PrP). When the TMD emerges, the N-terminus has not yet committed to the ER lumen, the TMD then has an opportunity to interact with the translocon and insert into the membrane. Chains that insert in the membrane become ^{Ctm}PrP, while chains that do not can become cyPrP (if the N-terminus is not translocated by the time synthesis is completed).

PrP: Prion protein; ^{sec}PrP: secretory prion protein; ^{Ctm}PrP: C-transmembrane form of prion protein which has the N terminus in the cytosol and C terminus in the ER lumen; ^{Ntm}PrP: N-transmembrane form of prion protein which has the N terminus in the ER lumen and C terminus in the cytosol; cyPrP: cytosolic form of prion protein; ER: endoplasmic reticulum; TMD: transmembrane domain.

Detailed analysis of this step has demonstrated that it is surprisingly complex and involves several factors.

First, signal sequences from different proteins carry out this step with markedly different efficiencies[51-52]. Second, this step involves interactions between the signal sequence and the central component of the translocation channel, Sec61 complex[53]. Third, the signal-translocon interaction appears to be influenced by at least two proteins termed the translocating-chain-associating membrane protein[54-55] and translocon-associated protein complex[56]. Fourth, not all signal sequences require translocating-chain-associating membrane protein and translocon-associated protein to function efficiently; while most (including prion protein) require at least one of these two complexes, a very small proportion of signal sequences can function well without either[53-54, 56]. The availability of endoplasmic reticulum lumenal chaperones appears to influence translocation[57-58], particularly prion protein. This pathway is a prerequisite for the generation of secretory prion protein or the N-transmembrane form of the prion protein, both of which have their N-terminus in the endoplasmic reticulum lumen. If this pathway is not followed, the N-terminus is not successfully translocated into the lumen, and remains in the cytosol. This can lead to the generation of the C-transmembrane form of the prion protein or cytosolic prion protein. In the post-translational pathway for prion protein insertion, a soluble pre-targeting complex captures the hydrophobic transmembrane domain of the tail-anchor substrate after it emerges from the ribosomal exit tunnel[59-60]. After loading onto Get3 (TRC40 in mammals[61]), the tail-anchor substrate is targeted to the endoplasmic reticulum membrane by interaction with Get1-Get2 receptor complex[62].

Thus, avoiding the generation of the C-transmembrane form of the prion protein and cytosolic prion protein requires the collective action of numerous determinants that include the signal sequence, Sec61 complex, TRAP complex, endoplasmic reticulum lumenal chaperones, and potentially yet unidentified factors.

AGGREGATION PRONE CHARACTERISTICS OF THE C-TRANSMEMBRANE FORM OF PRION PROTEIN AND CYTOSOLIC PRION PROTEIN

In general, protein aggregation is a common feature in

various diseases[63-65], and numerous mechanisms have been proposed by which protein aggregation can influence cellular physiology[66]. Remarkably, cytosolic aggregates also perturb the degradation of nontranslocated prion and membrane proteins[67]. Therefore, understanding the state of the minor specific neurotoxic molecule in some region-dependent contexts is likely to be of importance to fully understand the pathogenesis of prion diseases.

In yeast cells, even when using a native signal sequence from the yeast Kar2 protein, prion protein appears to be very inefficiently translocated into the endoplasmic reticulum, but also the non-glycosylated, non-disulfide bonded, cytosolic prion protein was prone to aggregation, insolubility, and partially resistant to protease digestion. These studies provided the basis for a provocative hypothesis that the cytosolic prion protein in mammalian cells could be the origin for the initial generation of scrapie-associated prion protein. Upon treatment of cells with proteasome inhibitors or overexpression of prion protein, a small proportion of mammalian prion protein could reside in the cytosol of mammalian cells and was found to be prone to aggregation, was insoluble in mild detergents, and at least partially resistant to protease digestion[39].

Several experiments have shown that to some degree, the ability of scrapie-associated prion protein accumulation to incite neurodegeneration is influenced by mutations that alter the ability of the host prion protein to transform into the C-transmembrane form[18-19]. The more easily the C-transmembrane form of prion protein can be generated, the more potent the effect of scrapie-associated prion protein, while the inability to generate the C-transmembrane form of the prion protein seems to confer some degree of protection from scrapie-associated prion protein accumulation[68].

PROTEIN QUALITY CONTROL IN THE CYTOSOL

In cells, the newly synthesized misfolded and mislocalized prion protein can perturb cellular homeostasis and provoke aggregation, pathological states, and even cell death. In order to survive, cells evolved powerful quality control strategies consisting of a large arsenal of molecular chaperones and proteolytic systems for repair[69] through removal of non-translocated populations of prion protein by the ubiquitin-proteasome system[34, 70], a proteasome-dependent pathway, or by macroautophagy selectively[71-72], which limit cytosolic prion protein levels at steady state. The pathway of their selective recognition, ubiquitination, and prompt degradation are essential for homeostasis pathways.

In general, failure to target the prion protein is typically due to intrinsic inefficiencies in targeting or translocation reactions[33], but may also be a consequence of regulated translocation[34]. For instance, Hegde and colleagues discovered that during acute endoplasmic reticulum stress, translocation of secretory and membrane proteins are rapidly and transiently attenuated in a signal sequence-selective manner[34]. Their cotranslational rerouting to the cytosol for degradation reduces the burden of misfolded substrates entering the endoplasmic reticulum and represents a pathway for pre-emptive quality control. The principal purpose of stress-dependent translocational regulation is to protect cells from excessive protein misfolding and aggregation in the endoplasmic reticulum[34, 37]. Conversely, improving signal sequence efficiency mitigated the effects of aggregates[67].

This series of events is spatially and mechanistically distinct from other pathways of proteasomal degradation[73] including failed targeting (in which nascent prion protein interacts with chaperones in the cytosol rather than signal recognition particles) or retrotranslocation (in which processed prion protein would be extracted from the endoplasmic reticulum lumen by the cytosolic VCP/p97 complex for degradation).

DIFFERENT DEGRADATION MECHANISMS OF THE CYTOSOLIC PRION PROTEIN AND THE C-TRANSMEMBRANE FORM OF PRION PROTEIN

Classical pathways of degradation

Because the ubiquitin-proteasome system is the most important pathway for selective protein degradation, especially for mislocalized prion protein, it is likely that this system is involved in the aetiology of neurodegenerative disorders. Indeed, ubiquitin-proteasome system impairment occurs during neurodegeneration[74]. Misfolded prion protein impairs the ubiquitin-proteasome system by interacting with the basal peptidase activity of 20S proteasome and inhibition of substrate entry[75].

An alternative pathway to the ubiquitin-proteasome system is autophagy, a lysosome-dependent degradation pathway conserved among all eukaryotes. Autophagy can degrade protein aggregates that cannot be transported to and unfolded by the proteasome. Autophagy

could serve as a compensatory degradation pathway when the proteasome is impaired and may cause proteasomal substrate degradation. On the other hand, severe proteasome impairment also impairs autophagy and *vice versa* provides evidence for the interdependence of autophagy and the ubiquitin-proteasome system[76]. Autophagy is essential for neuronal functioning because knock out of the UbL protein ATG5 results in neurodegeneration in the mouse brain and accumulation of ubiquitin-positive inclusions[77].

Emerman *et al* [43] used inhibitors to assess the normal degradation pathway used by the C-transmembrane form of the prion protein and cytosolic prion protein. Proteasome inhibition (with either MG132 or Lactacystin) clearly results in cytosolic prion protein levels exceeding the C-transmembrane form of the prion protein. By contrast, lysosomal inhibition with either Bafilomycin A1 or Leupeptin resulted in an increase in C-transmembrane form of prion protein relative to cytosolic prion protein. These results indicate that while cytosolic prion protein is primarily degraded by a proteasome-dependent pathway, the C-transmembrane form of prion protein is primarily degraded in lysosomes.

A novel mechanism: different sources of the minor prion protein bind to the same chaperone for degradation

The sources of cytoplasmic prion protein vary. Prion protein must be targeted in a correct and timely manner to the central portal of the secretory pathway, endoplasmic reticulum. However, translocation into the endoplasmic reticulum is an intricate, multistep process that involves protein targeting to the endoplasmic reticulum, translocon gating, energy-driven insertion of the protein through the endoplasmic reticulum membrane, and correct orientation of proteins relative to the membrane. It is not surprising that cytosolic prion proteins that remain in the cytosol are identified and sent off for degradation.

Bcl-2-associated athanogene family molecular chaperone regulator 6 is the product of a novel gene located within the human major histocompatibility complex[78] that encodes anti-apoptotic ubiquitin-like protein[79-80], and serves as the central component of a cytosolic three-protein complex. Bcl-2-associated athanogene family molecular chaperone regulator 6 is also a multitasker that ‘mops up’ mislocalized proteins with some common features for targeting and ubiquitination pathways[40]. In addition, it contains chaperone-like activity capable of maintaining an aggregation-prone substrate in an unfolded yet soluble state and a chaperone holdase capability[81].

Hessa *et al* [41] revealed that Bcl-2-associated athanogene family molecular chaperone regulator 6 was involved in the efficient ubiquitination of one kind of cytosolic prion protein after it had been released into the cytosol from the ribosome *via* exposed unprocessed or non-inserted hydrophobic domains. In contrast, another study has shown that for the substrate of endoplasmic reticulum-associated degradation, Bcl-2-associated athanogene family molecular chaperone regulator 6 and its cofactor Trc35 act as chaperones for retrotanslocated polypeptides en route to the proteasome to improve endoplasmic reticulum-associated degradation efficiency[41]. Endoplasmic reticulum-associated degradation employs a variety of chaperones and lectins such as BiP (also known as GRP78 and Kar2; protein chaperone glucose-regulated protein, 78 kDa), lectins Os9, α-mannosidase-like protein, and protein disulfide isomerase. They may selectively recognize terminally misfolded proteins carrying immature glycans, expose hydrophobic regions, or unpaired cysteine residues[69]. Compared with the first form of the Bcl-2-associated athanogene family molecular chaperone regulator 6 binding substrate, the endoplasmic reticulum-associated degradation molecule had been ubiquitinated on the endoplasmic reticulum membrane[82-83]. Some types of cytosolic prion proteins should also belong to the family of endoplasmic reticulum-associated degradation substrate, but the relationship between the specific cytosolic prion protein and the Bcl-2-associated athanogene family molecular chaperone regulator 6 complex remains unclear.

Overall, Bcl-2-associated athanogene family molecular chaperone regulator 6 is involved in mislocalized prion protein degradation depending on its ability to recognize the long linear hydrophobic stretches in such proteins in the cytosol.

CONCLUSIONS

Growing evidence has suggested that neurodegeneration may involve different aspects of prion protein metabolism beyond scrapie-associated prion protein. In addition, other isoforms of prion protein, such as cytosolic prion protein and C-transmembrane form of prion protein, may mediate neurotoxicity. However, further studies are required. It is important to identify other potential interacting partners of cytosolically exposed prion protein, and to clearly delineate their expression and function to elucidate how they might contribute to neurodegenera-

tion. For example, the central hydrophobic domain of prion protein (20 residues) that allows it to potentially span the membrane was found to be extremely well conserved across species[84]. They are absolutely invariant in all species including those as divergent as avians and reptiles (whose overall conservation is about 40% identity)[85]. This highly conserved, albeit unusual feature that is required for a proportion of prion protein to be a membrane-spanning protein suggested an alternative explanation for the transmembrane form. Perhaps the capability to synthesize transmembrane prion protein may be important for prion protein biology. Thus, cytosolic prion protein and the C-transmembrane form of prion protein are not intrinsically cytotoxic but depend critically on their cellular context. Furthermore, one explanation for the selectively of cell death in prion diseases may involve interacting partners, such as Mgrn and the Bcl-2-associated athanogene family molecular chaperone regulator 6 complex. Although their expression or functional importance is restricted, they play important roles in the ubiquitin-proteasome system. Finally, even though studies on the Bcl-2-associated athanogene family molecular chaperone regulator 6 complex have opened an exciting and new field of pre-insertional quality control, a specialized pathway for other kinds of cytosolic prion proteins lacking both the N-terminal targeting signal and the C-terminal glycosyl phosphatidyl inositol-anchoring signal must exist[86].

As more mechanistic insights are gained on other steps of prion protein biosynthesis and metabolism (endoplasmic reticulum associated degradation, the endoplasmic reticulum-resident protein-quality-control system and the ubiquitination-proteasome system), additional hypotheses and tools will be generated. The prompt ubiquitination and degradation mechanism, limiting the cytosolic prion protein levels at steady state and avoiding aggregate, is essential for cell homeostasis because mislocalized prion protein can make inappropriate interactions and cause neurodegeneration. These insights should be useful not only for the understanding of prion protein biology and the associated diseases, but also for uncovering novel cell biological principles. In analogous fashion, another idea with roots in prion protein biology and disease, that of information transfer mediated by protein elements, is now known to be far more generally applicable in other organisms and biological systems[86-87].

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