

## Chapter 16

# THE MOLECULAR BASIS OF PRION PROTEIN-MEDIATED NEURONAL DAMAGE

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### 16.1. Introduction

The most seductive questions in prion biology have always centered around the unusual nature of disease transmissibility<sup>1,2</sup>. What is the transmissible agent? What are the routes of transmission from one organism to another? How does the transmissible agent spread within an organism? What is the mechanism of replication? What is the nature of the barrier to transmission between species? Although the answers to these and related questions are far from clear, intense investigation over the past several decades has led to a working framework for understanding how the prion protein (PrP), in the absence of nucleic acids, could mediate the transmission and spread of neurodegenerative disease<sup>3-6</sup>.

By contrast, another series of questions has received far less attention within the prion field. This is in part because, rather than being specific and unique to prion diseases, this second set of questions is shared among a wide range of neurodegenerative diseases associated with abnormal protein accumulation. These include Alzheimer's, Parkinson's, and Huntington's diseases, among many others. The vital questions of interest in all of these areas concerns the molecular pathways that underlie the selective and devastating neuronal damage occurring during the pathogenesis of each of these diseases. Hence, one desires to know what the inciting event(s) or protein species are that lead to neurodegeneration. What are the causal relationships between protein aggregation and cellular toxicity? What pathways of cellular homeostasis are

disturbed and how does this lead to cell death? Why does pathology occur in only some but not other cell types?

It is this latter, poorly studied set of questions in prion biology that define the scope of this chapter. Unlike a traditional review article however, we will refrain from cataloging the bewildering array of toxicities, functions, and pathologies that have been ascribed to PrP over the years<sup>7,8</sup>. Such observations have often been conflicting, contentious, and limited in scope. Thus, an attempt to reconcile these claims, either with each other or to the pathogenesis of prion diseases is premature at the present time. We shall instead concentrate our efforts on delineating a systematic and logical *approach* for the study of PrP-mediated neurodegeneration. In developing these ideas, we have taken many cues from analogous studies of the other neurodegenerative diseases. Unencumbered by the issues of transmission, scientists in these other areas have delved more broadly and deeply into the mechanisms contributing to neuronal dysfunction during disease pathogenesis<sup>9-13</sup>. Conceptual lessons from these studies, although not specifically considered in this chapter, significantly shape the concepts articulated herein.

In any discussion of prion diseases, the issues of transmissibility (the first set of questions above) and neurodegeneration (the second set of questions) are inextricably linked. However, this linkage in thinking has significantly clouded meaningful investigations into the mechanism of neurodegeneration. In the first part of the chapter, we examine the basis for historically considering transmission and neurodegeneration as coupled aspects of disease pathogenesis. We then present the rationale, based on available studies, for asserting that the issues of neurodegeneration are not obligatorily coupled to transmission. And finally, we argue that for a productive investigation into PrP-mediated neurodegeneration, it should first be studied, both conceptually and experimentally, in isolation from the complexities of transmissibility issues.

In the second part of this chapter, we apply the ideas developed in the first half to formulating one way in which PrP-mediated neurodegeneration can be studied in the absence of PrP-templated transmissibility. We then present a framework for identifying and validating the molecular basis of neuronal damage during prion disease pathogenesis. In so doing, we will argue for the importance of a quantitative understanding of PrP cell biology, including its biosynthesis, trafficking, and metabolism. The best available information and candidates for PrP-mediated neuropathology will be discussed and the key steps for future studies aimed at their validation will be outlined. While the topic of discussion will be focused exclusively on diseases involving PrP, we hope that the general cell biological approach prescribed herein will provide a framework

within which to evaluate the pathogenesis of related neurodegenerative diseases that face remarkably similar issues once the complicating variables of prion transmissibility are stripped away.

## 16.2. Definitions

The nomenclature in the prion field is both heterogeneous and inconsistent. To avoid confusion, it is prudent to begin with clear definitions of the terminology used in this chapter. Transmissible spongiform encephalopathies (or TSEs) and prion diseases are synonymous: both refer to the spectrum of slowly progressing, *transmissible* neurodegenerative diseases with characteristic clinical and pathological sequelae<sup>2–6</sup>. The term prion, derived from the words **proteinaceous** and **infectious**, is *by definition* the transmissible agent in these diseases<sup>2</sup>. Importantly however, a specific molecular description of the prion (in terms of a precise composition) remains elusive. While most in the field would agree that a prion is free of large nucleic acids<sup>2–6</sup>, the potential presence or roles for small ligands, micro-RNAs, and other components have not been clearly defined. Thus, a prion is an agent, composed mostly if not exclusively of protein, responsible for the transmission of prion diseases (or TSEs) from one organism to another.

The prion protein (PrP), to be clearly distinguished from a prion, is the name for the most abundant and consistently identified protein in the purest available preparations of prions (and hence, the name originally given to this protein). This single protein has been described in numerous forms and locations. Thus, “PrP” is often appended with a modifier to more specifically refer to one particular species among several that include: PrP<sup>C</sup>, PrP<sup>Sc</sup>, C<sup>tm</sup>PrP, N<sup>tm</sup>PrP, <sup>sec</sup>PrP, cyPrP, PrP-res, and PrP-sen. In this chapter, the general term PrP is used to refer to this protein when no single form is being specified (e.g., as in PrP-mediated neurodegeneration). PrP<sup>Sc</sup> is used to specify the form that progressively accumulates during the course of prion disease and is therefore also the most abundant species in prions. This form is widely<sup>2–6</sup>, but not universally<sup>14–17</sup> thought to be the transmissible agent in prions. PrP<sup>C</sup> refers to the normal, endogenously expressed, cellular PrP: a GPI-anchored protein found on the surface of many cell types, most abundantly neurons. The terms PrP-res and PrP-sen, which define species of PrP on the basis of the biochemical property of protease resistance, will not be used in this chapter due to the ambiguity of what conditions distinguish ‘sensitivity’ versus ‘resistance’ to proteases. The remaining forms of PrP (<sup>sec</sup>PrP, C<sup>tm</sup>PrP, N<sup>tm</sup>PrP, and cyPrP) make distinctions based on the cellular locale and topological orientation of PrP relative to a membrane. Each of these is

defined in further detail in the subsequent sections where they are discussed.

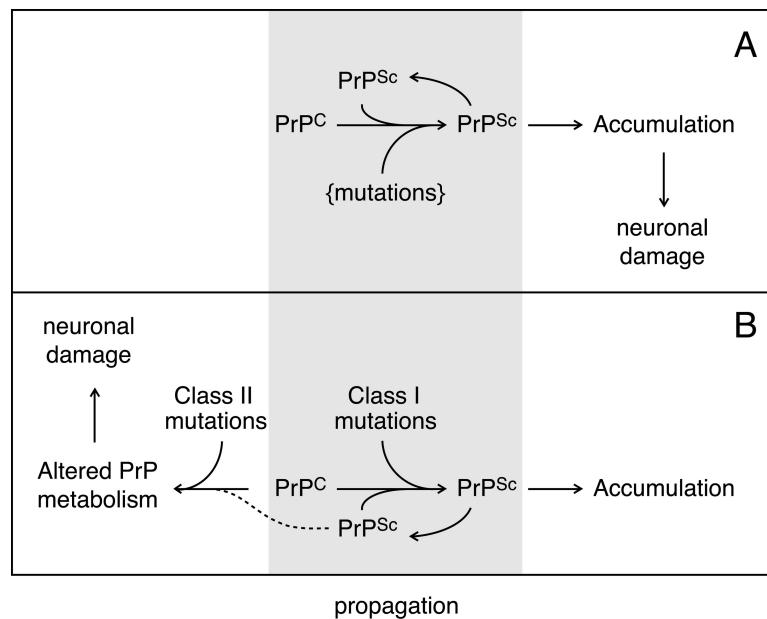
And finally, a word about the term pathogenesis. Since this term broadly encompasses all of the events that go awry during the course of a disease, it is often insufficiently specific in describing a particular facet of disease. In the case of the prion diseases, there are at least two distinct phases that we wish to distinguish: i) *transmission*—acquisition, replication, and accumulation of prions, and ii) *neurodegeneration*—the processes that result in the observed neuronal damage, pathology and clinical symptoms. Since the term pathogenesis includes both of these processes, we shall refrain from its use unless referring to the entire disease process. Otherwise, we shall use the more specific terms related to the aspect of disease pathogenesis under discussion.

### 16.3. Transmission versus neurodegeneration during prion disease pathogenesis

Prion diseases are by definition transmissible: they are either acquired by transmission and/or can be subsequently transmitted to another individual. This necessarily means that the disease involves the acquisition and replication of prions. It is entirely reasonable therefore to presume that the neurodegenerative disease that ensues must obligatorily be caused by the replication and accumulation of prions. Hence, the nature of the prion and the mechanisms underlying its replication would appear to be central questions in not only the transmissibility of prion diseases, but also in the neurodegeneration that results. For these reasons, an implicit assumption has generally been that an understanding of prion replication would also reveal the molecular basis of prion-mediated neurodegeneration. Unfortunately, this supposition has thus far not proven to be the case despite significant advances in defining both the nature of the prion and the mechanism of its propagation.

After identifying a protein,  $\text{PrP}^{\text{Sc}}$ , as the major component of prions<sup>2</sup>, a crucial advance was the subsequent discovery that this protein is encoded by a normal host gene<sup>18,19</sup>. The host encoded form of  $\text{PrP}$ , termed  $\text{PrP}^{\text{C}}$ , was found to be identical in sequence but distinct in conformation than  $\text{PrP}^{\text{Sc}}$  (ref. 20). These findings had two major consequences for the understanding of prion diseases. First, it immediately suggested a mechanistic model of prion propagation in which  $\text{PrP}^{\text{Sc}}$  would mediate the conformational conversion of  $\text{PrP}^{\text{C}}$  into additional copies of  $\text{PrP}^{\text{Sc}}$  (the so-called ‘protein-only’ or ‘prion’ hypothesis)<sup>2</sup>. In the two decades since this model was proposed, it has gained tremendous support and is the generally accepted paradigm of prion propagation and transmission<sup>3–6</sup>.

Figure 16.1. Two conceptually different ways (models A and B) of reconciling the relationship between transmissible and inherited forms of neurodegeneration mediated by PrP.



The second consequence of cloning the PrP gene was the resolution of a previously long-standing conundrum in prion diseases: how is it that a disease could have both familial and transmissible forms? The answer lies in the finding that the familial forms of these neurodegenerative diseases were caused by mutations in the gene encoding PrP (ref. 21–24). Thus, PrP can apparently cause disease in two ways. In transmissible forms of the disease, PrP<sup>Sc</sup> can induce the misfolding of host PrP<sup>C</sup> into additional copies of PrP<sup>Sc</sup>. Alternatively, mutations in host PrP can lead directly to disease.

In hindsight, these observations can be used to reconcile the transmissible and inherited forms of disease and unify them under a single paradigm in one of two (non-mutually exclusive) ways (Figure 16.1). The first possibility (model A) is that mutations in PrP destabilize its folding to predispose the spontaneous generation of the PrP<sup>Sc</sup> form. In this model, the spontaneously generated PrP<sup>Sc</sup> could then facilitate its further propagation from PrP<sup>C</sup>, accumulate, and cause disease by the same mechanisms involved in transmissible prion diseases. The second less obvious, but equally plausible possibility (model B) is that neuronal damage results from a derangement in some aspect of normal PrP<sup>C</sup> metabolism. In this view such altered cellular metabolism of PrP could be caused

either *directly* by certain PrP mutations, or *indirectly* as a secondary consequence of PrP<sup>Sc</sup> generation or accumulation. Thus, the most proximal cause of neurodegeneration is focused on PrP<sup>C</sup> in model B, but PrP<sup>Sc</sup> in model A.

Both models share several important features. First, the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> (indicated in gray shading) is the central event in the propagation and accumulation of PrP<sup>Sc</sup>, and therefore essential for *transmission* of disease. And second, both models posit that the transmissible and familial forms of the disease converge on a single proximal cause of neurodegeneration. Such a shared final pathway of neuronal damage would explain the similarities in clinical course and pathological findings common to the various neurodegenerative diseases mediated by PrP. Yet, the two views make dramatically different predictions about the key events leading to neurodegeneration. The first model proposes that the accumulation of PrP<sup>Sc</sup> not only generates more transmissible agent, but is the direct cause of neurotoxicity. By contrast, the second model proposes that some feature of PrP<sup>C</sup> metabolism causes neurodegeneration when misregulated (either directly by mutation, or indirectly by the accumulation of PrP<sup>Sc</sup>).

Historically, only the first of these two models has been articulated or seriously considered. The reason for this prejudice is because at the time of the identification of inherited PrP mutations, PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion was already well-established as the central event in both transmission and neurodegeneration. It was therefore logical and simpler to propose that PrP<sup>Sc</sup> accumulation acted *directly* to cause neuronal damage (model A) rather than *indirectly* via some other aspect of PrP metabolism (model B). In fact, a desire to experimentally discriminate between these two models was largely obscured by the strong bias in favor of the first model. This was further complicated by the rarity of the human genetic forms of these diseases, making their molecular analysis difficult. Thus, the two views have never been systematically or directly tested for their validity. Instead, an *ad hoc* series of experiments, often designed for other purposes, must be evaluated to help discriminate between the different views of PrP-mediated neurodegeneration.

One of the key predictions of model A is that human neurodegenerative diseases caused by mutations in PrP should accumulate PrP<sup>Sc</sup>, generate transmissible agent (i.e., prions), and should therefore be transmissible. Testing definitively for PrP<sup>Sc</sup> or prion accumulation in diseased human tissue is complicated by several confounding variables. While protease resistance and relative insolubility provide valuable surrogate markers of PrP<sup>Sc</sup>, neither of these properties is unique to PrP<sup>Sc</sup> (that is, there are several ways to make PrP insoluble or protease resistant without necessarily generating disease-associated PrP<sup>Sc</sup>; see for example,

ref. 25). Conversely, the lack of these properties would not necessarily rule out the presence of  $\text{PrP}^{\text{Sc}}$ , especially since digestion and solubilization conditions are operationally defined and variable from one laboratory to another. Nonetheless, such analyses have been done on at least some of the familial cases of PrP-mediated neurodegenerative disease with mixed results. Some mutations (e.g., D178N, E200K, or V210I; ref. 26 and 27) result in the abundant accumulation in brain of protease-resistant PrP whose digestion properties and fragments are characteristic of  $\text{PrP}^{\text{Sc}}$ . Other mutants (e.g., P102L or F198S) appear to accumulate PrP in a state distinguishable from normal  $\text{PrP}^{\text{C}}$ , but do not have the same biochemical properties of  $\text{PrP}^{\text{Sc}}$ . For example, they may be only partially resistant to proteases, or yield digestion fragments of different sizes<sup>28–35</sup>. Yet other mutants (e.g., A117V) show no obvious biochemical evidence of  $\text{PrP}^{\text{Sc}}$  accumulation<sup>33,34</sup>.

Meanwhile, the most definitive test for prions, a bioassay, was being performed in parallel on comparable samples<sup>36–39</sup>. However, due to a potential species barrier between the source (human) and host (usually rodents, or in some instances non-human primates), the interpretation of transmission results are often complicated. In these experiments, disease transmissibility appears to correlate more or less with the biochemical studies: samples showing clear evidence of  $\text{PrP}^{\text{Sc}}$  (e.g., E200K) transmit disease with high frequency, while others (e.g., P102L or A117V) transmit disease to few or no recipients. While these observations further strengthen the case for  $\text{PrP}^{\text{Sc}}$  as the transmissible agent in prions, it was also the first indication that neurodegeneration could potentially be caused by PrP mutants that did not obligatorily generate either  $\text{PrP}^{\text{Sc}}$  or prions. It was therefore at least feasible that the development of PrP-mediated neurodegenerative disease could be uncoupled from the replication and accumulation of prions (i.e., suggested by model B).

Despite these results, the experimental difficulties and the inability to do conclusive studies without the confounding variables of species barriers left a definitive conclusion out of reach. It remained entirely possible that prions did form and accumulate in all of these PrP-mediated diseases, but were simply not readily detectable in some instances by the admittedly complicated assays being employed. Thus, the simplest view remained that *both* transmission and neurodegeneration depend absolutely on the replication and accumulation of prions, a process presumed to be synonymous with the conversion of  $\text{PrP}^{\text{C}}$  to  $\text{PrP}^{\text{Sc}}$  (i.e., model A). Indeed, an alternative view was essentially still not considered. What then was imagined to be the cause of neurodegeneration? Two obvious (non-mutually exclusive) possibilities were usually cited. Either neurodegeneration was a result of the depletion of  $\text{PrP}^{\text{C}}$  as a

consequence of its conversion to  $\text{PrP}^{\text{Sc}}$ , or the accumulation in brain of the insoluble, aggregation-prone  $\text{PrP}^{\text{Sc}}$  form was proposed to be inherently harmful. However, both of these possibilities soon proved difficult to demonstrate.

The first surprising finding was the observation that PrP knockout mice are phenotypically normal<sup>40</sup>. As predicted, these mice are resistant to prion infection and propagation<sup>41,42</sup>, consistent with a requirement for endogenous  $\text{PrP}^{\text{C}}$  in the replication of  $\text{PrP}^{\text{Sc}}$ . However, the fact that the absence of  $\text{PrP}^{\text{C}}$  did not directly cause neurodegeneration suggested that the depletion of  $\text{PrP}^{\text{C}}$  during prion replication may not be the cause of neurodegeneration. At the time, it remained possible that the knockout mice, having lacked PrP from the single-cell stage, did not mimic the acute  $\text{PrP}^{\text{C}}$  depletion that might occur during prion disease. However, recent studies in which no adverse consequences were observed upon post-natal disruption of the PrP gene strongly argue against the depletion of  $\text{PrP}^{\text{C}}$ , either acutely or chronically, as the cause of neurodegeneration<sup>43,44</sup>.

This then left the proposed toxicity of  $\text{PrP}^{\text{Sc}}$  accumulation as the most obvious candidate in causing neurodegeneration. Unfortunately, the simplest variant of this hypothesis quickly became untenable as well. In a wonderfully elegant experiment, the brain tissue of PrP-expressing mice was grafted into the brain of PrP-knockout mice<sup>45</sup>. Upon inoculation of these grafted mice with prions, the PrP-expressing tissue replicated the prions and generated large amounts of  $\text{PrP}^{\text{Sc}}$  and transmissible agent. Despite its deposition throughout the brain, only the tissue actively expressing PrP succumbed to neurodegeneration; the PrP-knockout tissue remained completely unaffected even after prolonged exposure to  $\text{PrP}^{\text{Sc}}$  and transmissible prions. This conclusion has been confirmed more recently by a completely independent approach in which PrP was selectively depleted in neurons after the initiation of prion infection<sup>44</sup>. Even though the infection continued to generate prions and  $\text{PrP}^{\text{Sc}}$  (presumably via non-neuronal cells such as astrocytes), the neurons remained free from further damage. In fact, the degeneration present at the time of PrP depletion may even have been reversed at later points. Thus, it appears that the accumulation of  $\text{PrP}^{\text{Sc}}$  is not inherently toxic to neurons *per se*.

From these various observations, it has become increasingly clear that the cause of neurodegeneration in prion diseases cannot easily be explained by the most apparent events that accompany the replication of prions: the acute depletion of  $\text{PrP}^{\text{C}}$  or the accumulation of  $\text{PrP}^{\text{Sc}}$ . Instead, these observations suggest remarkably that the accumulation of  $\text{PrP}^{\text{Sc}}$  and of prions is neither necessary (e.g., in the case of some familial PrP mutants) nor sufficient (e.g., in the context of neurons not

actively expressing PrP<sup>C</sup>) for neurodegeneration. Yet, the evidence that conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> is the central event in prion replication and disease transmission is now overwhelming<sup>3–6</sup>. How then can these two conclusions be reconciled?

The simplest way would be to posit that the events that are of paramount importance to transmission (PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion) are not necessarily the same ones that are critical for neurodegeneration. Clearly however, both facets of the disease involve host-encoded PrP, but apparently in different ways. For prion replication and transmission, host PrP is absolutely required as a source of substrate for PrP<sup>Sc</sup> propagation. For neurodegeneration (in both transmissible and non-transmissible forms of disease), ongoing PrP expression in the cells that eventually succumb to disease is absolutely required. *Thus, the primary insight that we currently have into the basis of neurodegeneration in these diseases is that some aspect of active PrP expression or metabolism is required for its selective toxicity to neurons.* For these reasons, we argue that while historically, there have been good reasons to consider transmissibility and neurodegeneration as coupled events in prion disease pathogenesis, the fact that they can be uncoupled experimentally and naturally merits their consideration as separate and distinct phases of the disease. But should neurodegenerative processes be studied separately from transmissibility and prion replication, and if so, how?

#### **16.4. The case for uncoupling neurodegeneration from transmission**

There are several reasons, both conceptual and technical, to study the neurodegenerative processes of PrP-mediated disease independently of transmission. A particularly pragmatic reason relates to the biochemical properties of PrP<sup>Sc</sup> that make the cell biological and biochemical study of other PrP isoforms difficult. First, the half-life of PrP<sup>Sc</sup> in cultured cells or brain tissue is substantially longer than other PrP species<sup>46,47</sup>, resulting in its much higher levels at steady state. Second, PrP<sup>Sc</sup> is both highly aggregated and heterogeneous<sup>48,49</sup>. Together, with its high abundance during transmissible disease pathogenesis, these properties make biochemical fractionation of the various PrP isoforms exceedingly difficult. Thus, nearly all fractions of any separation method contain amounts of PrP<sup>Sc</sup> that are comparable to or exceed other PrP isoforms. Third, sensitive reagents (e.g., antibodies) highly specific to the PrP<sup>Sc</sup> form remain elusive despite extensive efforts and some claims of success<sup>50–52</sup>. Thus, its definitive and high resolution detection in

individual fractions, within a cell (e.g., by immunofluorescence), or in tissue remain difficult. Fourth, PrP<sup>Sc</sup> is generally highly resistant to protease digestion relative to the other forms of PrP. This, combined with the lack of specific antibodies for PrP<sup>Sc</sup> make it difficult to remove selectively in instances where the other PrP isoforms need to be analyzed. And finally, PrP<sup>Sc</sup> continues to elude a clear molecular or structural description. Many different 'strains' have been identified<sup>53</sup> that differ in poorly defined ways with respect to both transmissible and biochemical properties.

It is therefore clear that the presence and accumulation of PrP<sup>Sc</sup> during PrP-mediated neurodegeneration makes the selective analysis of non-PrP<sup>Sc</sup> forms of PrP difficult. This has been a principal reason that any role in neuronal damage for PrP isoforms other than PrP<sup>Sc</sup> has been difficult to evaluate. Conversely, such non-PrP<sup>Sc</sup> forms are relatively easily removed (for example, with protease digestion) to selectively reveal the more abundant PrP<sup>Sc</sup>. While this has facilitated PrP<sup>Sc</sup> analysis during transmissible disease progression, it has also obscured other PrP forms that may contribute to or cause neurodegeneration. Thus, an evaluation of any role for non-PrP<sup>Sc</sup> forms in the development of neuronal damage would be facilitated greatly by systems in which PrP-mediated neurodegeneration is recapitulated in the absence of PrP<sup>Sc</sup> accumulation.

A second reason to uncouple the neurodegenerative from transmissible phases of disease relates to the multi-factorial and complex parameters that influence transmission of prion diseases<sup>3–6</sup>. These factors include the 'strain' of prion involved, the passage history (i.e., in what species did it pass through), the primary sequences of the host versus exogenous PrP, yet undefined factor(s) needed for prion replication (e.g., a hypothetical protein X, among other factors), and incompletely defined modifiers of prion susceptibility and incubation time<sup>54–58</sup>. These parameters each influence the time course of the disease, the pathological features that are observed, and the cell-type specificity of involvement. Ideally, it is desirable to simplify these variables by analyzing disease in a model where a defined inciting event (such as a point mutation) leads as directly as possible to the pathway of neurodegeneration without influencing too many other events that would obscure the relevant pathogenic steps.

The third reason to study the later neurodegenerative steps in the absence of transmissible agent is the simple practicality of biosafety and containment. The study of prions in either cell culture or mouse models requires specific biosafety considerations that involve a substantial investment of resources. Equipment and space are generally dedicated to prion work, making their use for other studies impractical. This makes

it difficult for investigators in other fields of study to initiate prion-related studies. However, the issues of PrP-mediated neurodegeneration, if recapitulated in the absence of transmissible agent, can be studied as any other cell-biological or pathological process. Since these downstream events are likely to involve aspects of basic cell biology, signal transduction, apoptosis, etc., their analysis would be markedly facilitated by the involvement of experts in these different fields. Thus, models of PrP-mediated neurodegeneration in isolation from the issues of transmission would reduce barriers to a multi-disciplinary approach to these problems. As argued in the previous section, there are now compelling reasons to believe that in fact, PrP-mediated neurodegeneration is not obligatorily linked to either PrP<sup>Sc</sup> or the formation and accumulation of transmissible prions. Therefore, it is not only feasible, but desirable to experimentally uncouple these two phases of the disease to facilitate the mechanistic dissection of the neurodegenerative process.

### **16.5. Genetic prp-mediated neurodegeneration as a model system**

What is the most productive way to study the neurodegenerative phase of PrP-mediated disease in the absence of PrP<sup>Sc</sup> or transmissible agent? We believe the answer to this question lies in a careful consideration of model B (Figure 16.1). In this model, prion disease pathogenesis is depicted in two phases: the replication and accumulation of prions, followed by the neurodegeneration induced by this process. Both phases are experimentally known to require ongoing PrP synthesis, but appear to involve different aspects of its metabolism. In the first phase (shaded in gray), PrP<sup>C</sup> is needed as the substrate for the template-mediated conversion into PrP<sup>Sc</sup>, an event thought to be essential for the generation of prions. In the second phase, the role of PrP expression is unknown, but is an absolute prerequisite for neuronal cell death. Thus, some feature of PrP metabolism, after its active *de novo* synthesis, is altered in a way that leads to neurodegeneration. The neurodegenerative events are initiated in one of two ways: either as an indirect consequence of PrP<sup>Sc</sup> formation and accumulation during transmissible prion disease (indicated by the dotted line in model B), or due to inherited mutations in the PrP gene in genetic disease.

In the case of the genetic diseases, the mutation can be envisioned to act in one of two ways. In the first way (designated Class I; see Figure 16.1), the mutation may influence PrP<sup>C</sup> folding in a manner that facilitates its spontaneous conversion to PrP<sup>Sc</sup>. Once this spontaneous event occurs, PrP<sup>Sc</sup> would mediate its templated self-propagation to not

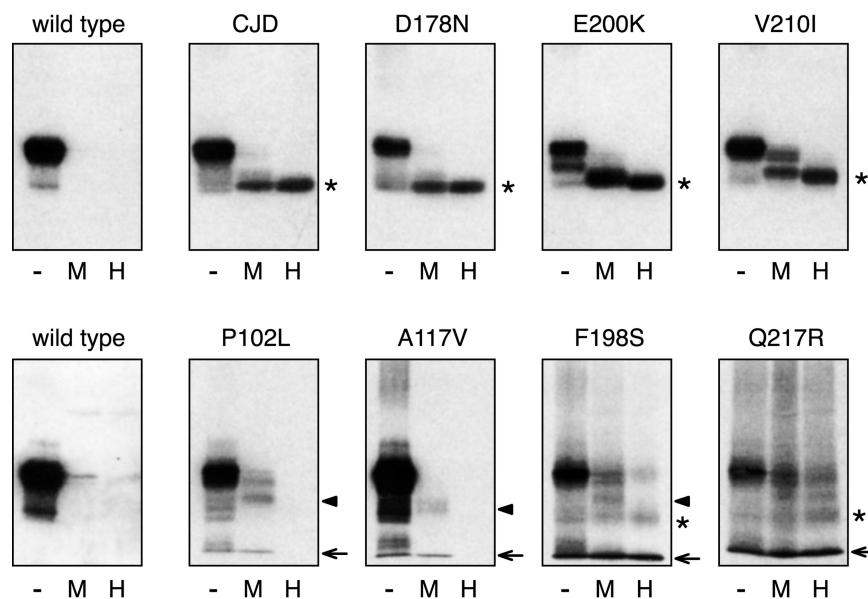
only generate more PrP<sup>Sc</sup>, but to initiate the heretofore unknown events leading the neurodegeneration. Thus, these forms of genetic disease act by first generating PrP<sup>Sc</sup> (and hence, are predicted to be transmissible) which then leads to neurodegeneration by the same mechanisms utilized in transmissible prion diseases. The second way PrP mutations could cause disease is to alter PrP metabolism in a way that recapitulates its ability to cause neuronal damage. In these instances (designated Class II), the effect is directly on PrP<sup>C</sup> metabolism and therefore need not involve PrP<sup>Sc</sup> formation. Thus, such inherited diseases would neither accumulate PrP<sup>Sc</sup> nor be transmissible.

The phenotype of Class I PrP mutants, if recapitulated in model systems, would be ideal for studying PrP<sup>Sc</sup> formation and replication. Here, a defined change in primary sequence facilitates not only spontaneous conversion to PrP<sup>Sc</sup>, but its subsequent self-propagation in a way that reconstitutes disease pathogenesis. By contrast, Class II mutations should allow the steps of PrP-mediated neurodegeneration to be reconstituted in a model system *without the involvement of PrP<sup>Sc</sup>*. Clearly, the most insight into neurodegeneration with the least confounding variables would be to study such genetic lesions that bypass prion replication and directly modulate the aspects of PrP metabolism that initiate the molecular pathways leading to neuronal dysfunction and death. Thus, an important step lies in distinguishing between the two ways that inherited PrP mutations lead to disease, and identifying for study those that are likely to work by the second mechanism.

Simplifying the study of a complex, multi-factorial disease process by first focusing on genetic examples that may recapitulate key facets of pathogenesis is an approach with numerous important precedents. This is analogous to progress in other complex diseases such as Alzheimer's disease or breast cancer. In these instances, the analysis of rare genetic variants were instrumental in illuminating particular molecular players and mechanistic steps to guide a better understanding of the more commonly occurring forms of disease that were otherwise too heterogeneous to allow systematic analysis. Even though clinical and pathological features of the genetic variants often differ in significant ways from the non-heritable forms of the disease, the initial faith that they would all share at least some common mechanistic steps at the molecular level was eventually validated upon further study.

At this stage in our understanding of prion disease pathogenesis, a similar faith is needed in the commonality of the underlying mechanistic steps involved in the different disease variants. Clearly, there are many differences among the various genetic, sporadic, and transmissible forms of PrP-mediated diseases. Yet, they all share the involvement of PrP, certain pathologic features, their late onset followed by rapid disease progression, and the selective involvement of the central

Figure 16.2. Different biochemical properties of PrP among various disease-associated mutations. Brain tissues from the indicated human diseases were analyzed before ('-') or after protease digestion using mild ('M') or harsh ('H') conditions (as defined in ref. 33). Tissue from non-familial CJD was also analyzed in parallel. All samples were digested with PNGase to remove glycans prior to analysis by immunoblotting.



nervous system despite widespread PrP expression. For these reasons, a faith in at least some shared common features is probably not misguided, and more than offset by the potential for simplification by studying select inherited examples of PrP-mediated neurodegenerative disease. Even if the pathogenic events are not shared among the genetic and transmissible forms of disease, at the very least, insight into the pathogenic events of a model protein folding disease will be illuminated by studying PrP mutants that directly cause neurodegeneration.

There are roughly 30 choices among the genetic lesions in PrP that cause neurodegeneration<sup>59,60</sup>. The vast majority of these very rare mutations have not been studied in any significant detail. In at least a few instances however, sufficient analysis has been performed to evaluate whether they might be Class I or II mutants of PrP. Since one primary distinction between Class I and Class II mutants is whether they accumulate PrP<sup>Sc</sup>, a biochemical analysis is perhaps the simplest way to initially categorize the mutants. As one such example (see Figure 16.2, which essentially recapitulates previous observations<sup>26-35</sup>), the susceptibility of PrP to different protease digestion conditions is evaluated. Here, a very high proportion of total PrP from transmissible CJD

is characteristically resistant to 'harsh' protease digestion in a manner that generates *only* a resistant C-terminal domain (indicated by an asterisk in Figure 16.2). This behavior indicates that the majority of PrP is in the PrP<sup>Sc</sup> form at the time of death from illness. Exactly this behavior, *both quantitatively and qualitatively*, is observed for several of the human PrP mutants (e.g., E200K, D178N, and V210I), suggesting that like transmissible disease, these familial forms also accumulate large amounts of PrP<sup>Sc</sup>. Indeed, these are also the heritable diseases that have been easily transmitted to animal hosts with high efficiency<sup>36-39</sup>. Thus, they appear to be Class I mutants that may work by favoring the spontaneous generation of PrP<sup>Sc</sup>.

By rather striking contrast, several other heritable PrP mutants show distinctly different behaviors. These mutants do not result in the accumulation of PrP forms whose C-terminal domain is highly resistant to 'harsh' protease digestion. Instead, some (such as P102L or A117V) appear to contain PrP forms that are only 'mildly' resistant to protease digestion (arrowheads in Figure 16.2). Others contain and accumulate smaller metabolic fragments of PrP (arrows, Figure 16.2) that seem to resist protease digestion and are not observed at comparable levels in normal brain tissue. These observations suggest that these mutants may cause a change in some aspect of PrP metabolism, but does not generate much if any PrP<sup>Sc</sup>. Consistent with this interpretation, brain homogenates from such samples do not appear to transmit disease to experimental animals<sup>36-39</sup>, suggesting that they may represent Class II mutations that directly lead to neurodegeneration in the absence of either transmissible agent or PrP<sup>Sc</sup>. Similar observations of altered metabolism without apparent PrP<sup>Sc</sup> or prion accumulation has also been made with other more gross mutations in PrP. These additional putative Class II mutants include premature stop codons and octapeptide (or octarepeat) insertions into the N-terminal domain of PrP (ref. 61-65). Hence, as a group, Class II mutants would appear to be the best candidates for examples of disease in which PrP metabolism is directly and specifically affected to cause neurodegeneration, without the complicating feature of prion replication and accumulation. Among them, point mutants may represent the best choices for a model system since their effects are presumably more selective than large insertions or premature truncations.

## 16.6. The importance of PrP cell biology

How can one use the genetic lesions that are hypothesized to selectively cause neurodegeneration to understand the mechanistic basis of the disease process? There are two qualitatively different, non-mutually

exclusive directions. The first approach is to reconstitute the key events of cellular dysfunction that accompany neurodegeneration in a model system amenable to experimental manipulation. The second strategy involves comparative quantitative analyses of the cell biological behavior and metabolism of PrP and its disease-associated variants to generate testable hypotheses for the mechanisms involved in initiating neuronal damage. The rationale, utility, and current progress toward both of these strategies are discussed in turn below, with our argument for why the second, cell biological approach is particularly important at the present stage of progress in this field.

The first approach, that of selectively reconstituting PrP-mediated neuronal damage in a model system, would allow the evaluation and dissection of the steps leading from a defined lesion in PrP to eventual cell death. Ideally, the system of choice would be the simplest and most manipulable model, such as yeast or perhaps cultured cells that allow the combined use of genetic, molecular, and biochemical tools to easily modulate individual gene products, cellular pathways, and environmental parameters. For such a system to be useful, it must recapitulate at least some facets of the native disease process, such as the selective effects of known disease-associated mutations. At this point, no such model has been successfully developed or validated. This contrasts sharply with the process of PrP<sup>Sc</sup> propagation, which has been both reproduced in several cell culture systems<sup>66–69</sup> and validated by the demonstration of infectivity in animal bioassays<sup>69</sup>. Such systems have proved quite valuable over the past 15 years in helping to uncover features important to PrP<sup>Sc</sup> generation and propagation.

The difficulties of recapitulating the key features of neuronal dysfunction in simplified systems are many. These include the apparently extreme cell-type specificity of this process *in vivo*. Only neurons appear to be obviously affected during disease<sup>70–71</sup> despite widespread expression of PrP in multiple cell types both in and out of the nervous system<sup>72–73</sup>. Furthermore, specific and different subsets of neurons are affected in the different disease variants with no clear explanation<sup>3–8,74,75</sup>. These observations indicate that very precise cellular conditions that may not be easily recapitulated in other model systems (such as yeast or cultured cell lines) are necessary to manifest the downstream consequences associated with PrP mutations or PrP<sup>Sc</sup> accumulation. In addition, the cellular context may play a currently unappreciated key role. For example, the *in vivo* situation of multiple interacting cell types and defined external cues such as hormones, growth factors, or extracellular matrix may substantially influence PrP-mediated neurotoxicity in ways that are difficult to reproduce in culture.

A second problem is that *in vivo*, the disease is temporally confined both in terms of the slow progression and defined age of onset. The basis of these observations is not known; why is it that despite expressing a mutant PrP gene at high levels for between 40–50 years, the disease is only manifest late in life? Thus, faithfully reproducing neurodegenerative events in a simplified system stripped of the *in vivo* context and under vastly different time scales, while potentially very useful and appealing, may be daunting. Tricks of PrP overexpression and the use of stressful conditions to tax the cells may be necessary to facilitate PrP-mediated toxicity in such model systems; however, such manipulations may make distinguishing effects of the normal from mutant proteins especially difficult. Furthermore, without specific intermediate markers of PrP dysfunction, one would need to reconstitute the entire downstream set of events that lead to detectable cellular damage. This may encompass too many steps to easily accomplish in simplified cellular systems. These considerations help to define the obstacles that one must consider in the establishment of model cellular systems, and perhaps explain why such systems have been very slow to develop in both PrP-mediated and other neurodegenerative diseases.

Many of these obstacles could potentially be overcome by the use of whole organisms that contain multiple differentiated cell types in which the likelihood of recapitulating the desired neurodegenerative pathways is increased. Again, one would seek to observe effects that are selective to both neurons and disease-associated PrP mutants. Although a homologue of PrP is not found in the genomes of either *Drosophila* or *C. elegans*, these are both attractive candidates for such an approach due to their genetic manipulability and the existence of tools for large scale loss-of-function screens using RNAi methodology. At present, little effort has been expended towards these goals, largely due to the focus on the transmissible features of the disease that have long been reconstituted in a cell culture system. Indeed, in the case of polyglutamine expansion-mediated protein aggregation and neurodegeneration (where transmissibility is not an issue), useful models have been developed and exploited in both *C. elegans* and *Drosophila*<sup>76–78</sup>. Similar models for the neurodegenerative phase of PrP-mediated disease should greatly facilitate both the testing of hypotheses related to the pathogenic events (see below) and the elucidation of the cellular pathways involved. Until such simpler model systems are developed and validated, one must either work within the confines of the modestly manipulable, slow time frames characteristic of transgenic mice, or take parallel alternative strategies to obtaining mechanistic insights into PrP-mediated neurodegeneration.

The principal parallel strategy that, in our opinion, offers the highest likelihood of success is founded on a thorough and quantitative

understanding of PrP cell biology. In short, the logic is that in order to understand the causative basis of a disease-associated PrP mutation, the metabolism of the mutant PrP needs to be compared to and distinguished from wild type PrP in simplified biochemical and cell culture systems. In this manner, one can identify potential differences in the behavior of PrP mutants that may account for their biological consequences *in vivo*. It is important to note that in these experiments, the mutant PrP is not anticipated to necessarily induce the eventual consequences of cell damage in the model system. As discussed above, the downstream pathways are not likely to be easily recapitulated. Rather, this approach is intended to identify differences between wild-type and mutant PrPs that would represent *potential* initiating events for subsequent neurodegenerative sequelae. Once specific points of difference are identified in the pathways of PrP metabolism, hypotheses can be formulated regarding the role of such events in the neurodegenerative process. The readily manipulable biochemical and cell biological systems employed to initially identify the differences in PrP metabolism should also facilitate the development of tools to exaggerate or minimize the key step(s) in question. Finally, such tools would then be used in suitable model systems (such as transgenic mice) to either validate or negate hypotheses that propose key roles in inciting neurodegeneration. Thus, in this strategy, basic aspects of PrP cell biology are studied in easily manipulated and rapidly analyzed systems to generate hypotheses that are subsequently tested in the more laborious and slow *in vivo* setting only after specific tools and mechanistic insights are available.

A key step in this experimental strategy is to determine in molecular detail the cell biological properties and metabolism of PrP to facilitate its comparison to the mutants. This is not necessarily to learn the normal function of PrP, since loss of its still unknown functions<sup>43</sup> are not thought to be the key event leading to neurodegeneration. Rather, it is to facilitate the identification of an apparently dominant, gain of function feature imparted by the mutant that is likely to cause neurodegeneration. Put another way, how can one possibly figure out what goes 'wrong' without a clear description of what 'right' looks like? Thus, in the absence of a quantitative description of the steps in PrP biosynthesis and metabolism, one cannot reasonably hope to detect anything but the most dramatic effects caused by mutant PrP variants. However, dramatic consequences of PrP mutations are not particularly likely in light of the fact that the disease manifests over such a prolonged time frame. This principle is analogous to the effects of mutations in Alzheimer's precursor protein (APP) that cause neurodegeneration. The mutations do not have overtly obvious effects on APP metabolism; rather, most of them subtly influence specific processing events involved in the generation of

a particular peptide fragment (a-beta) which, over many decades, has adverse consequences<sup>9</sup>. Such effects would have been very difficult to detect without well-defined and quantitative assays for the normal events in APP metabolism. Similar and analogous parallels can be drawn with many other slowly-developing diseases in which small biochemical effects are sufficient to cause disease over appropriate time scales in the correct *in vivo* context. It is therefore imperative that *in vitro* analyses should have the capability to quantitatively detect subtle differences in a variety of parameters related to PrP cell biology.

What then are the facets of PrP cell biology that should be the focus of our attention? Given that the normal function of PrP is neither known nor is believed to play a role in neurodegeneration, it is most reasonable to dissect the steps in PrP biosynthesis, maturation, trafficking, and degradation pathways. These metabolic events seem particularly relevant in light of the observation that even in cases where PrP<sup>Sc</sup> accumulation is not occurring, various other forms of PrP are often deposited as plaques or other aggregates at the later stages of the disease<sup>70,71</sup>. While the causative role of such deposits remains uncertain, they do indicate that some facet of its normal metabolism has gone awry at some point during pathogenesis. Thus, the operant questions regarding PrP that should be asked and addressed include: what are the key steps during its biosynthesis? What machinery is required for its proper entry into the endoplasmic reticulum (ER)? What factors in the ER are involved in its modifications, folding, and maturation? How efficient are these various steps during its biogenesis? What happens to the population of PrP molecules that fail in their maturation? What are PrP's different destinations in the cell? How is it trafficked to these sites such as the cell surface, and what additional maturation steps occur en route? What are the pathways and time frame for its normal turnover? What machinery is involved, and what metabolic products are generated?

Thus, each facet of the life of PrP from its point of synthesis to its recycling into degradative products should be analyzed quantitatively. By analyzing these same events for various disease-associated mutations, specific steps that may be deranged, even very slightly, can be identified. Once these potential differences are found, they can then be studied to investigate the mechanistic basis of the effect and subsequently modified to either enhance or decrease the process in question. These tools can then be used *in vivo* to test specific hypotheses regarding the pathogenesis of prion diseases. While the complete sequence of investigation is far from complete in any aspect of PrP metabolism, some initial studies have identified potential candidates for being involved in PrP-mediated neurodegeneration. Below are described the historical development and current state of investigation into these aspects of PrP cell biology, their

relationship to neurodegeneration, and some comments on what is now required to further our understanding.

### **16.7. *C<sup>tm</sup>PrP and the development of neurodegeneration***

When the gene encoding PrP was first cloned, an obvious (and deceptively simple) question was to determine its normal biosynthetic pathway and cellular locale. Since PrP<sup>C</sup> (and PrP<sup>Sc</sup>) were known to be glycosylated, PrP was presumed to be trafficked through the secretory pathway. Indeed, sequence analysis of the full length PrP open reading frame suggested an N-terminal signal for targeting to the ER and two potential sites for N-linked glycosylation in the C-terminal domain. In addition, the sequence revealed a hydrophobic domain of ~20 residues and a downstream amphipathic region. These elements were incorporated together into a model of PrP as a double-spanning transmembrane protein in which the N- and C-termini were in the lumen<sup>79</sup>. Such a model was supported by the initial analysis of PrP topology upon its *in vitro* synthesis using wheat germ extracts and ER microsomes derived from canine pancreas<sup>80</sup>.

The view of PrP as a transmembrane protein was very short lived. It was quickly realized that when synthesized in a *mammalian* translation system (rabbit reticulocyte lysate) with pancreatic ER microsomes, PrP was fully translocated across the membrane (similar to a secretory protein, and hence the operational designation of this topologic form as 'secretory'-PrP or <sup>sec</sup>PrP)<sup>81</sup>. Furthermore, 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<sup>82</sup>. Thus, the 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 PrP is a GPI-anchored cell surface glycoprotein. All other observed forms were thought to represent either mistakes or artifacts (and hence, irrelevant to normal PrP function). This is the view that generally persists today.

Curiously however, the transmembrane form of PrP, while exaggerated in the wheat germ system (>80% of total PrP, depending on translation conditions), is nonetheless also observed (at an albeit lower level of ~5–10%) in the reticulocyte lysate system<sup>33</sup>. This topological heterogeneity had not been observed for any of numerous model secretory

or membrane proteins that had been examined in the *in vitro* translocation systems: not only were these other proteins made faithfully in their predicted topology, but the same outcomes were obtained in the wheat germ, reticulocyte, and cell culture systems. Furthermore, the central hydrophobic domain of PrP that allows it to potentially span the membrane was subsequently found to be extremely well conserved across species<sup>83</sup>. These ~20 residues are absolutely invariant in all species including those as divergent as avians and reptiles (whose overall conservation is ~40% identity)<sup>84,85</sup>. This highly conserved, albeit unusual feature that is required for a proportion of PrP to be made as a membrane-spanning protein<sup>86</sup> suggested an alternative explanation for the transmembrane form. Perhaps the capability to make transmembrane PrP (at least under some conditions) may be both normal and important for some aspect of PrP biology. Unfortunately, the lack of a clear functional role for PrP made this hypothesis difficult to explore. Furthermore, a related idea that transmembrane PrP could somehow play a role in disease generally was not considered because at that time, the much more dramatic observation of PrP<sup>Sc</sup> accumulation suggested a more obvious culprit.

However, several concurrent studies began to suggest that while PrP<sup>Sc</sup> formation was clearly associated with disease transmission, its accumulation was not inherently toxic to neurons. The first hint was the observation that when mice heterozygous for the PrP gene (PrP<sup>+/−</sup>) were inoculated with prions, PrP<sup>Sc</sup> accumulation followed a course very similar to that observed in wild type mice (i.e., PrP<sup>+/+</sup>). Yet, the progression to clinical disease resulting from neurodegeneration was markedly delayed<sup>87</sup>. This discordance between PrP<sup>Sc</sup> and neuronal damage was particularly dramatic in brain grafting experiments<sup>45</sup> where PrP knockout neurons appeared impervious to any adverse consequences of PrP<sup>Sc</sup> deposition. In parallel, the identification of the PrP gene made possible the discovery of a wide range of inherited PrP mutations that led to familial forms of PrP-mediated disease<sup>59</sup>. Biochemical analyses of tissue from such familial cases (e.g., as in Figure 16.2) suggested that while some of them had accumulated PrP<sup>Sc</sup>, others were surprisingly devoid<sup>26–35</sup>. Such biochemical results were, over the course of several years, corroborated by extensive transmission studies<sup>34–39</sup>. Thus, by the mid-1990s, it was reasonable to consider the possibility that PrP-mediated neurodegeneration could be caused by means other than through PrP<sup>Sc</sup> accumulation.

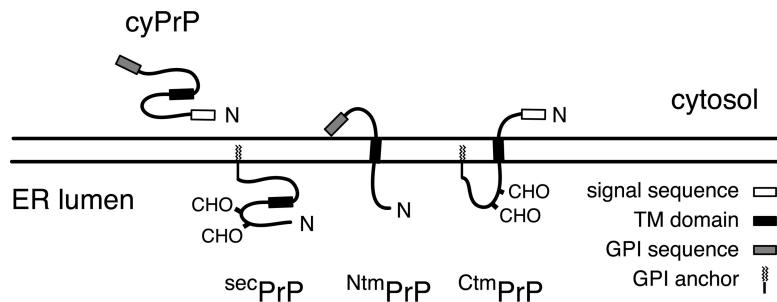
It was in the context of these studies that the question of the proximal causes of neurodegeneration was brought into slightly better focus. A reconsideration of a possible role for the originally observed transmembrane form of PrP was stimulated by the discovery that at least

one disease-associated mutation (A117V) and a disease-influencing polymorphism (at codon 129) were in the highly conserved domain of PrP predicted to form a potential transmembrane segment. How then could one examine if and how this transmembrane form might play a role in disease? First, in preliminary experiments, it was observed that in fact, the A117V mutation influenced PrP biogenesis: a very subtle, but reproducibly detectable increase in transmembrane PrP (from ~5–10% to ~10–15%) was observed in translocation assays carried out in reticulocyte lysates. Motivated by this *in vitro* observation, it was then hypothesized that perhaps PrP-mediated neurodegeneration could be caused by transmembrane PrP. Indeed, it had been observed for some time that while PrP<sup>C</sup> is largely released from the cell surface by cleavage of its GPI anchor, PrP<sup>Sc</sup> remained cell-associated even after GPI anchor cleavage<sup>88</sup>. While this had many interpretations, one possibility was that PrP<sup>Sc</sup> is membrane anchored by another mechanism, perhaps via a transmembrane topology. Unfortunately, the biochemical obstacles to analyzing PrP<sup>Sc</sup> precluded a direct examination of this hypothesis.

Therefore, a different tact was taken: mutations that favor or disfavor the generation of the transmembrane form to different extents would be expressed in transgenic mice lacking their endogenous PrP to observe the consequences, if any, for neurodegeneration. Using the *in vitro* translocation assay, mutations (in or around the central hydrophobic domain of PrP) were assayed for their effect on topology. In the course of these studies, it was realized that rather remarkably, there was not one, but two transmembrane forms that were being generated<sup>33</sup>. One spanned the membrane with the N-terminus in the ER lumen and C-terminus in the cytosol, while the other was in exactly the reverse orientation. These were dubbed <sup>Ntm</sup>PrP and <sup>Ctm</sup>PrP, respectively (see Figure 16.3). Most of the mutations that increased the transmembrane forms in these original studies seemed to preferentially increase <sup>Ctm</sup>PrP (ref. 33, 35), and hence for somewhat arbitrary reasons, this became the form of interest while the <sup>Ntm</sup>PrP form has thus far been poorly studied.

When expressed in transgenic mice on a PrP-null background, PrP mutants that favor transmembrane forms of PrP (and in particular <sup>Ctm</sup>PrP) caused the development of neurodegenerative disease<sup>33,35</sup>. Upon the compilation of several such mutants (that ranged from ~10% to ~50% <sup>Ctm</sup>PrP generation, as compared to ~5% for wild-type), it became clear that there was a dose-response effect: the more heavily <sup>Ctm</sup>PrP is favored or the more highly expressed the <sup>Ctm</sup>PrP-favoring transgene, the earlier the development of neurodegeneration. Conversely, two different mutations that reduce or abolish the ability to generate either of the transmembrane forms did not lead to neurodegeneration<sup>33</sup> (although for reasons that are not yet clear, transgenic lines expressing these

**Figure 16.3.** Depiction of the different topologic forms of PrP:  ${}^{\text{sec}}\text{PrP}$ ,  ${}^{\text{Ctm}}\text{PrP}$ ,  ${}^{\text{Ntm}}\text{PrP}$ , and cyPrP. At present, there remains some uncertainty regarding whether cyPrP and  ${}^{\text{Ctm}}\text{PrP}$  contain uncleaved signal sequences<sup>33,89–91</sup>. Although an uncleaved signal can be detected on at least some  ${}^{\text{Ctm}}\text{PrP}$  and cyPrP chains when they are generated by overexpression in cultured cells<sup>89,91</sup>, it is less clear whether this is also the case under normal circumstances *in vivo*. Of these forms,  ${}^{\text{sec}}\text{PrP}$  and  ${}^{\text{Ctm}}\text{PrP}$  contain a C-terminal GPI anchor and two N-linked glycans<sup>33,89</sup> that are not found on either cyPrP or  ${}^{\text{Ntm}}\text{PrP}$ .



mutants proved more difficult to stably maintain). Thus, with the accumulated data from four different  ${}^{\text{Ctm}}\text{PrP}$ -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  ${}^{\text{Ctm}}\text{PrP}$  and neurodegenerative diseases<sup>33,35</sup>.

Biochemical analyses of brains from these transgenic mice revealed the presence of  ${}^{\text{Ctm}}\text{PrP}$  (ranging from ~5–30% of total PrP) in those lines that both expressed  ${}^{\text{Ctm}}\text{PrP}$ -favoring mutants and were prone to neurodegeneration<sup>33</sup>. Again, a dose-response relationship was observed between  ${}^{\text{Ctm}}\text{PrP}$  in brain and severity of the neurodegenerative phenotype<sup>33,35</sup>, validating in many ways the *in vitro* translocation systems in which these mutants (as well as the transmembrane forms themselves) were first identified. In a particularly satisfying experiment, one of the PrP constructs whose expression in mice led to neurodegeneration was the human disease-associated A117V mutant<sup>35</sup>. Even more remarkably, human tissue from such a patient contained detectable amounts of  ${}^{\text{Ctm}}\text{PrP}$  but not  $\text{PrP}^{\text{Sc}}$  (ref. 33). Indeed, this was one of the genetic diseases that was particularly puzzling because it was caused by a derangement in PrP, and yet was neither transmissible<sup>34</sup> nor contained  $\text{PrP}^{\text{Sc}}$ . Consistent with these original observations in humans, transgenic mice with  ${}^{\text{Ctm}}\text{PrP}$ -mediated neurodegeneration lack  $\text{PrP}^{\text{Sc}}$  (ref. 33, 35). In extensive attempts at transmission involving hundreds of recipients,  ${}^{\text{Ctm}}\text{PrP}$ -mediated neurodegeneration was shown to be non-transmissible<sup>35</sup>.

These results established that inappropriate generation of  $^{Cm}PrP$ , even at only slightly elevated levels beyond wild type PrP, could result in neurodegeneration without the obligate generation of either  $PrP^{Sc}$  or infectious prions. Such a mechanism of PrP-mediated neurodegeneration was likely involved in at least two of the naturally occurring PrP mutants leading to heritable disease (P105L, in addition to A117V, leads to increased  $^{Cm}PrP$  generation)<sup>31</sup>. With the finding of at least one mechanism by which an alteration of PrP cell biology (in this case, its initial biogenesis at the ER) can cause disease, a more complicated question was raised: what role if any does  $^{Cm}PrP$  play in the pathogenesis of transmissible diseases? Addressing this issue, which presently remains unresolved, will be difficult and requires considerably more mechanistic knowledge about the biology of  $^{Cm}PrP$ . However, some potential insight into this issue can be obtained from the experiments performed thus far.

To begin examining the potential role for  $^{Cm}PrP$  in the neurodegeneration caused upon accumulation of  $PrP^{Sc}$  during transmissible disease, the various lines of  $^{Cm}PrP$ -altering transgenic mice were utilized in a different way. In these experiments, the susceptibility of each of the transgenic mouse lines to prion inoculation was assessed<sup>35</sup>. The logic was that if  $^{Cm}PrP$  was involved in the neurodegeneration caused by  $PrP^{Sc}$ , then modulating the propensity for  $^{Cm}PrP$  to be generated (by either favoring or disfavoring it with mutations in the transmembrane domain) should influence the progression of disease. By contrast, if  $PrP^{Sc}$  accumulation caused neuronal damage by mechanism(s) not involving  $^{Cm}PrP$ , then slight alterations in the ability to generate this form should have no effect on disease. Put another way, the experiment aimed to test whether in transmissible prion disease, the neurodegenerative phenotype correlated better with  $PrP^{Sc}$  accumulation or  $^{Cm}PrP$ -generating potential.

Despite numerous caveats and potential confounding variables, the experiment yielded a surprisingly clear result: increased propensity of PrP to be made in the  $^{Cm}PrP$  form sensitized mice to developing neurodegeneration during transmissible prion disease<sup>35</sup>. A particularly good illustration of this effect can be seen when mice expressing the A117V mutation (which very slightly favors  $^{Cm}PrP$ ) are compared to the  $\Delta$ STE mutation (which decreases, although does not completely eliminate the generation of transmembrane forms of PrP). Here, the two lines of mice express the transgene at equal levels in a PrP-null background. Upon inoculation with prions, the A117V mice develop neurodegeneration in less than 60 days, at a time when only a relatively small amount of  $PrP^{Sc}$  has accumulated. By marked contrast, the  $\Delta$ STE mice do not develop signs of neurodegeneration for up to 350 days after inoculation. By this point,  $PrP^{Sc}$  has accumulated to levels more than 5–10 times that

observed in the A117V mice at the time they became ill. Mice expressing wild type PrP at comparable levels get sick at an intermediate time of  $\sim$ 100 days<sup>54</sup>. Hence, it appeared that the ability of PrP<sup>Sc</sup> accumulation to incite neurodegeneration is influenced by mutations that alter the ability of host PrP to be made in the <sup>Ctm</sup>PrP form<sup>35</sup>: the more easily <sup>Ctm</sup>PrP can be generated, the more potent the effect of PrP<sup>Sc</sup>, while the inability to generate <sup>Ctm</sup>PrP seems to confer some degree of protection from accumulated PrP<sup>Sc</sup>.

One expectation from such a model relating <sup>Ctm</sup>PrP to PrP<sup>Sc</sup> accumulation is that during the course of transmissible disease, the amount of total <sup>Ctm</sup>PrP should rise (since its elevated levels are what is postulated to be the cause of neurodegeneration). Examining this idea directly poses a substantial technical hurdle because during the course of disease, the levels of PrP<sup>Sc</sup> also rise dramatically. Since PrP<sup>Sc</sup> at later points of disease progression is very abundant, highly heterogeneous in its fractionation properties, relatively insoluble, and protease resistant, the possibility of detecting small changes in <sup>Ctm</sup>PrP seem slim. Indeed, one would anticipate that only a small increase in <sup>Ctm</sup>PrP would be necessary to cause neurodegeneration given that some of the heritable disease mutations elevate <sup>Ctm</sup>PrP only slightly. In an attempt to circumvent such technical hurdles, double-transgenic mice expressing PrP from two different species were employed.

In this experiment, mice expressing both mouse PrP and hamster PrP were inoculated with mouse prions<sup>35</sup>. Given the species barrier to transmission<sup>54</sup>, it was expected that only mouse PrP<sup>Sc</sup> would be generated and accumulate. The hamster PrP, for which specific antibodies exist, would serve as a 'reporter' for measuring <sup>Ctm</sup>PrP. The question being asked was whether the accumulation of PrP<sup>Sc</sup> (of mouse origin) leads to some change in host PrP metabolism that results in increased <sup>Ctm</sup>PrP (measured by examining the hamster PrP 'reporter'). Although indirect in its approach, a slight ( $\sim$ 2–3 fold) increase in <sup>Ctm</sup>PrP was detected<sup>35</sup>. Indeed, based on the heritable mutations in both mice and humans, a mere 2-fold increase in <sup>Ctm</sup>PrP generation is potentially significant since it is clearly sufficient to cause neurodegeneration<sup>33,35</sup>. Thus, a working hypothesis, albeit based on indirect and complex experiments, is that the increased generation of <sup>Ctm</sup>PrP represents a step that is common to several types of PrP-mediated neurodegenerative diseases including the transmissible variety. One of the most important features of these studies and this hypothesis is that it provides a toehold into at least one direct cause of neurodegeneration, makes specific predictions about its generality, and is readily testable (as discussed further in section 9).

We therefore feel that at the present time, the generation of <sup>Ctm</sup>PrP is perhaps the most specific and well-defined event in PrP cell biology

that has been directly linked to causing neurodegeneration. Indeed it remains the only proposed model that identifies a very specific neurotoxic molecule (<sup>Ctm</sup>PrP), delineates the site (the ER) and mechanistic steps that can lead to its increased generation, demonstrates its presence *in vivo*, and tightly correlates its elevated presence in both experimental and naturally occurring PrP-mediated disease. Many of the other potentially toxic molecules (such as various fragments of PrP<sup>28–30,92,93</sup>, or incompletely defined misfolded forms<sup>94</sup>) and events (such as PrP crosslinking at the cell surface<sup>95</sup>, or increased PrP retention in the ER<sup>91,96</sup>) that have been proposed as a cause of neurodegeneration have yet to meet all (or in some cases, any) of these same criteria. Until this is achieved, sufficiently specific hypotheses and precise experimental tools cannot be generated to yet merit a serious consideration of their proposed roles in prion disease pathogenesis.

### **16.8. cyPrP and the development of neurodegeneration**

Recently, a cytosolic form of PrP (cyPrP) has been discovered and suggested to play a role in PrP-mediated neurodegeneration. In this example, a specifically defined species of PrP has been identified, demonstrated to at least be *capable* of causing neurodegeneration, and mechanism(s) for its generation *in vivo* have been proposed based on well-established cellular pathways<sup>90,97</sup>. Thus, while a role for cyPrP in prion disease pathogenesis is even less established and more contentious than for <sup>Ctm</sup>PrP, sufficiently specific and testable hypotheses have been formulated to merit its careful consideration (reviewed in ref. 98).

The idea that PrP, which is normally co-translationally targeted to and translocated across the ER membrane, can reside in the cytosol has its genesis in *Saccharomyces cerevisiae*. It is ironic (and perhaps meaningful) that just as transmembrane PrP was initially discovered as a likely 'artifact' of expression in a heterologous wheat germ system, cyPrP was also first noticed when expression of mammalian PrP was attempted in the yeast system. In yeast cells, PrP appears to be very inefficiently translocated into the ER, even when a native signal sequence from the yeast Kar2 protein is used<sup>99</sup>. While this is perhaps not very surprising, what led to further investigation was the finding that the non-glycosylated, non-disulfide bonded, cytosolic PrP was prone to aggregation, insoluble, and partially resistant to protease digestion<sup>90,99</sup>. Although proteins in the wrong cellular compartment of a non-native organism are often misfolded, the superficial resemblance between PrP aggregates in the yeast cytosol and PrP<sup>Sc</sup> in mammalian prion disease

provided the basis for a provocative hypothesis<sup>90,99</sup>: perhaps even in mammalian cells, PrP in the cytosol could be the origin for the initial generation of PrP<sup>Sc</sup>.

This hypothesis then raised the important questions of whether in mammalian cells, PrP (or disease-associated mutants) can ever reside in the cytosol, and if so, what relevance this would have for either PrP<sup>Sc</sup> formation or disease pathogenesis. The issue of whether PrP can potentially reside in the cytosol was initially addressed indirectly by demonstrating that in cultured cells overexpressing PrP, a small proportion of it was degraded by a pathway that could be inhibited by proteasome inhibitors<sup>90,100,101</sup>. Thus, upon treatment of cells with such inhibitors, an unglycosylated, presumably cytosolic form of PrP 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 PrP could reside in the cytosol of mammalian cells.

Based largely on co-migration in SDS-PAGE with recombinant PrP lacking a signal or GPI anchoring sequence, it was thought that cyPrP had been subjected to processing by ER-luminal signal peptidase and GPI-anchoring machinery<sup>92</sup>. Thus, cytosolic PrP was suggested to have originated from ER localized PrP that had failed to be properly folded<sup>92</sup>. In this view, the well-established (albeit incompletely understood) ER-associated degradation pathway<sup>102</sup> was being utilized by PrP molecules that had failed to meet the cellular quality control systems<sup>103</sup> in the ER lumen. It would then be retrotranslocated from the ER to the cytosol, deglycosylated by cytosolic N-glycanase, and degraded by the proteasome pathway. Hence, inhibition of the proteasome would cause accumulation of the species to be degraded, thereby explaining the appearance of cytosolic, unglycosylated PrP under these conditions.

The most compelling aspect of these studies, especially as it relates to disease, was the observation that a disease-associated PrP mutation (D178N) was found to a higher extent in the cytosol than wild type PrP under both normal and proteasome-inhibited conditions<sup>90</sup>. The supposition, which remains largely untested at present, was that this mutation was less likely to fold properly in the ER and therefore result in a higher proportion of it utilizing the ER-associated degradation pathway. This idea was especially attractive because it could potentially apply to many if not all disease-associated PrP mutants to provide a common mechanism for their adverse effects.

In order to provide support to these ideas, it was important to first provide proof of principle for two important predictions of the hypotheses linking cyPrP to prion diseases. First, if cyPrP is in fact involved in the de novo generation of PrP<sup>Sc</sup> and/or transmissible prions, better

evidence was needed in addition to rather non-specific biochemical features such as aggregation and protease resistance. And second, a role for cyPrP in neurodegeneration cannot even be considered without at least demonstrating that it *can* cause selective damage to neurons. The first charge was approached by attempting to determine if the most central feature of PrP<sup>Sc</sup>, its self-propagation using host-encoded PrP<sup>C</sup>, could also be demonstrated for cyPrP. In these experiments<sup>101</sup>, an initial 'seed' of cyPrP was initiated by transient treatment of PrP-expressing cells with proteasome inhibitor. Then, the inhibitor was removed to determine whether this seed of cyPrP could grow (i.e., propagate itself) by recruitment of additional PrP molecules. Exactly such a phenomenon was observed, and used to support the proposition that at least one source of *de novo* PrP<sup>Sc</sup> formation could be the cytosol<sup>101</sup>. Additional support is provided by the finding that the D178N mutant has both increased residence in the cytosol of cultured cells<sup>90</sup> and spontaneous PrP<sup>Sc</sup> generation in human patients<sup>26,37</sup>.

At present however, it remains to be seen whether the cyPrP aggregates are in fact transmissible and propagated when introduced into animals. Furthermore, the controls for complete removal of the proteasome inhibitor in the 'seeding' experiments were not particularly compelling since an unrelated protein did not resume degradation (although it did not accumulate like PrP)<sup>101</sup>. In addition, alternative interpretations are possible in which the apparent propagation is due to continued inhibition of the proteasome (at least partially) by the cyPrP aggregates in a manner observed for other non-transmissible proteins<sup>104</sup>. And finally, artifacts of overexpression in heterologous systems have also been suggested as an explanation<sup>91</sup>. Thus, while one interpretation of the data involves the spontaneous conversion of cyPrP to PrP<sup>Sc</sup> in the cytosol, this remains unproven. However, the idea is readily testable by the appropriate infectivity assays using both cell culture derived cyPrP aggregates as well as material from brain tissue expressing cyPrP (see below).

The second prediction, that cyPrP is toxic to neurons, was demonstrated directly when PrP was forced to be expressed in the cytosol by removal of its N-terminal signal sequence (and C-terminal GPI anchoring sequence). In both cultured cells of neuronal origin and certain subsets of neurons in transgenic mice, forced expression of cyPrP resulted in neurodegeneration<sup>97</sup>. Thus in at least some (but clearly not all<sup>97,105,106</sup>) neurons under certain conditions, cyPrP *can* be detrimental. These results now provide sufficient key elements to propose a testable framework for neurodegeneration in prion diseases involving cyPrP<sup>97,99,101</sup>.

In this model, a small proportion of PrP is always transiently trafficked through the cytosol prior to its rapid degradation by cytosolic

proteasomes. This transient cytosolic population would be increased with either mutations (as in the case of heritable disease) or alterations that perturb the proteasome degradation pathway. Such perturbations would be postulated to result from  $\text{PrP}^{\text{Sc}}$  accumulation, old age, or both. If cyPrP accumulates above a certain threshold, it would not only cause cell death, but aggregate into a form that has self-perpetuating capability. This self-perpetuating aggregate, once released from the cell, would recruit PrP from the surface of other cells to generate the observed glycosylated  $\text{PrP}^{\text{Sc}}$  that is seen in prion diseases. While numerous questions remain unanswered in this rudimentary framework, it, like the ideas centered around  $\text{C}^{\text{tm}}\text{PrP}$ , draw heavily upon basic cell biological pathways to make specific and testable predictions regarding their respective roles in disease pathogenesis. Indeed, it is also quite plausible that the two sets of ideas share a common mechanistic feature given that in both models, a key feature involves exposure of at least a portion of PrP to the cytosolic environment.

### 16.9. Testing the roles of $\text{C}^{\text{tm}}\text{PrP}$ and cyPrP in prion disease

In section 16.6, ('The importance of PrP cell biology'), we argued that a quantitative and mechanistic understanding of PrP cell biology may be the most productive way to identify facets of PrP metabolism that have potential importance for prion disease pathogenesis. Through such studies, we felt that insights and tools would be generated to allow selective manipulation of these steps in PrP metabolism *in vivo* to test the consequences for neurodegeneration. Where along this prescribed path do the studies of  $\text{C}^{\text{tm}}\text{PrP}$  and cyPrP stand, and what are the best future directions?

At this point, the relatively easy part has been accomplished. Rather drastic *exaggeration* of some facet of PrP metabolism with a fairly blunt manipulation (such as deleting the signal sequence in the case of cyPrP) has been used to cause disease in a whole organism<sup>33,35,97</sup>. By striking contrast, the far more difficult task will be to selectively *reduce* the propensity for this same event *in vivo* to test its possible role during prion infection and pathogenesis. Accomplishing this goal will either require tremendous luck, or a significant degree of mechanistic insight into the molecular pathways involved in the respective aspects of normal PrP cell biology. For example, reducing the propensity for PrP to ever be in the cytosol would need sufficient insight into the pathways by which it is routed there normally so that one could selectively modulate this event. Such modulation is required to rigorously test whether the ability

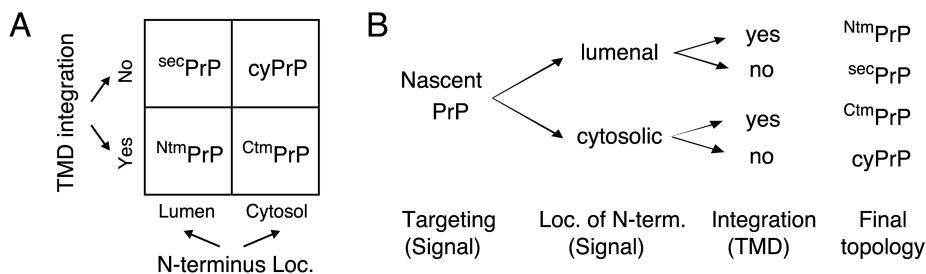
of PrP to be in the cytosol is important for the cell death that occurs during PrP<sup>Sc</sup> accumulation. Such experiments would also help examine the (non mutually exclusive) proposal that PrP in the cytosol is actually protective during prion disease<sup>105,106</sup>.

It should therefore be clear that in order to productively move forward in the <sup>Ctm</sup>PrP and cyPrP fields, two directions merit a high priority at the present time. First, the pathways for the generation, trafficking, and degradation of these molecules needs to be understood in mechanistic detail. Second, and of slightly lower initial priority, the interactions between these molecules and specific cellular pathways needs to be defined. During the course of these studies (particularly the first aim), valuable tools will emerge with which to selectively modulate the synthesis, metabolism, or function of <sup>Ctm</sup>PrP and cyPrP. Such tools can then be applied to precisely probe the complex problem of neurodegeneration during prion disease pathogenesis. Little progress has been made towards the second aim; the proteins that interact with either <sup>Ctm</sup>PrP or cyPrP, the pathways they influence, or the way in which they cause cell death all remain totally unknown. Fortunately, mechanistic studies of the biogenesis of <sup>Ctm</sup>PrP (and to a lesser extent, cyPrP) have begun to yield some insights which should facilitate their modulation *in vivo*.

After recognizing that PrP can be made in at least three distinct topological forms<sup>33</sup> (four if one includes cyPrP), a framework was needed to understand how a single polypeptide could acquire multiple outcomes during its synthesis at the ER. A key realization was that the topologic forms (see Figure 16.3) differ in two important ways. The first is the location of the N-terminus: either in the cytosol (as for cyPrP and <sup>Ctm</sup>PrP) or in the ER lumen (<sup>sec</sup>PrP and <sup>Ntm</sup>PrP). The second is whether the central hydrophobic domain becomes membrane integrated (<sup>Ntm</sup>PrP and <sup>Ctm</sup>PrP) or not (<sup>sec</sup>PrP and cyPrP). Each of the four combinations of these two 'decisions' describes uniquely each of the four topologic outcomes (schematically depicted in Figure 16.4A). For example, <sup>sec</sup>PrP results from the decision to have the N-terminus translocated into the ER lumen, and a decision to not integrate the potential transmembrane domain into the membrane. In this model, heterogeneity at one or both of these decisions would lead to the generation of multiple topologic forms of PrP (see Figure 16.4B). Thus, to understand the basis of PrP biogenesis, it is crucial to decipher the mechanism by which these two decisions are made and regulated by the cell.

To begin addressing this issue, a mutational analysis was carried out to determine which domains of PrP are involved in localization of the N-terminus and membrane integration<sup>107</sup>. These experiments revealed that localization of the N-terminus (cytosol versus ER lumen) is influenced largely by the N-terminal signal sequence. Mutations in the signal

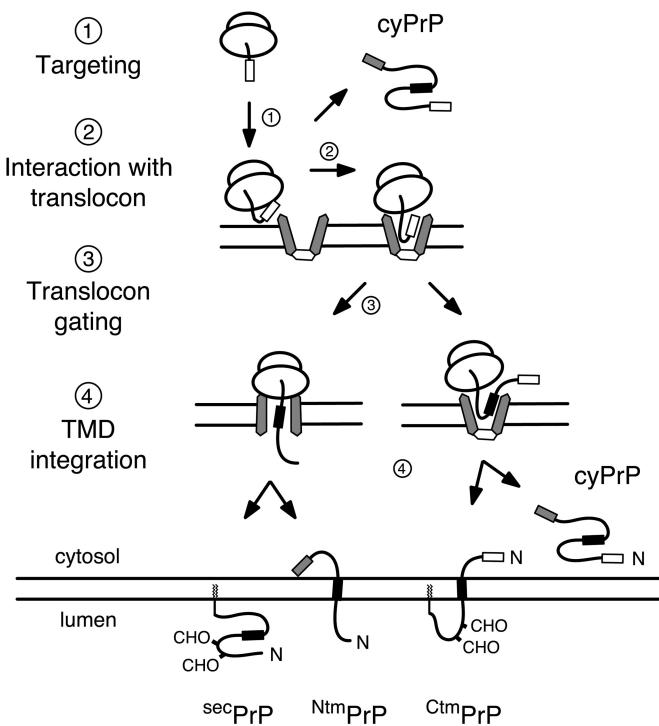
**Figure 16.4.** Schematic depiction of PrP topogenesis at the ER. Two decisions are combined to determine the final topologic outcome for PrP. The two decisions and four potential outcomes are depicted in chart format in Panel A and sequential order in Panel B. The first decision involves the N-terminal signal sequence and determines whether the N-terminal domain of PrP will be in the cytosol or ER lumen. The second decision involves the potential TMD, and determines whether the protein will be integrated into the membrane or remain soluble.



sequence could be identified which increase cytosolic localization (and hence, increase  $C^{tm}\text{PrP}$  and  $cy\text{PrP}$  relative to  $N^{tm}\text{PrP}$  and  $sec\text{PrP}$ ) or increase luminal localization (and hence, decrease  $C^{tm}\text{PrP}$  and  $cy\text{PrP}$ ). By contrast, the second decision regarding membrane integration is influenced largely by the highly conserved potential transmembrane domain (TMD). In this case, changes which (even slightly) increase hydrophobicity of the TMD result in increased generation of the membrane integrated forms ( $C^{tm}\text{PrP}$  and  $N^{tm}\text{PrP}$ ) relative to the non-integrated forms ( $cy\text{PrP}$  and  $sec\text{PrP}$ ). Thus, the signal sequence and TMD act together to allow the potential generation of four distinct topologic forms of PrP.

Experiments analyzing serial intermediates of increasing nascent chain length during PrP synthesis have revealed the sequential order of events involved in the determination of PrP topology<sup>108</sup> (Figure 16.5). These experiments demonstrated that the first step is 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 (SRP) and SRP-receptor pathway<sup>109</sup>. Targeting to the ER appears to be essential for the generation of  $C^{tm}\text{PrP}$ ,  $N^{tm}\text{PrP}$ , and  $sec\text{PrP}$ ; in the absence of a functional signal sequence, PrP is made exclusively in the cytosol<sup>108</sup>. After targeting, but before the TMD is synthesized and emerges from the ribosome, there is a brief window of time during which a particularly critical step takes place. During this step, 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 lumen, a prerequisite for the generation

Figure 16.5. A mechanistic depiction of the key steps during PrP biogenesis at the ER.



of  ${}^{\text{sec}}\text{PrP}$  and  ${}^{\text{Ntm}}\text{PrP}$ . 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 the ribosome-nascent chain complex remains in close proximity to the translocon while the remainder of PrP is synthesized.

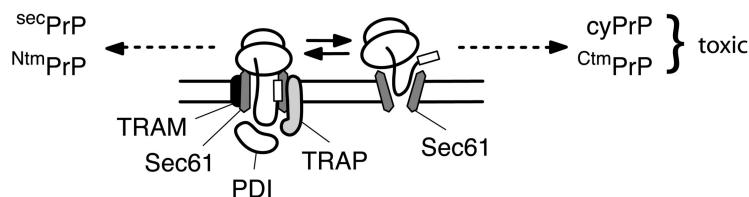
As this key step is occurring, the TMD is synthesized and emerges from the ribosome. If the N-terminus has already been committed to the ER lumen, determinants in the TMD (primarily hydrophobicity) influence the propensity of the chain to become membrane integrated (to generate  ${}^{\text{Ntm}}\text{PrP}$ ) or fully translocated (to become  ${}^{\text{sec}}\text{PrP}$ ). If, when the TMD emerges, the N-terminus has not been committed to the ER lumen, the TMD then has an opportunity to interact with the translocon and become inserted into the membrane. Chains that insert in the membrane become  ${}^{\text{Ctm}}\text{PrP}$ , while chains that do not can become cyPrP (if the N-terminus is not translocated by the time synthesis is completed). Mutational analysis suggests that one key determinant of this TMD-mediated integration step that generates  ${}^{\text{Ctm}}\text{PrP}$  is hydrophobicity<sup>107,108,110</sup>. This appears to explain the mechanism of increased  ${}^{\text{Ctm}}\text{PrP}$  generation for

at least some disease-associated PrP mutants (e.g., A117V, P105L, and most recently, G131V)<sup>31,34,111</sup> that increase hydrophobicity of the TMD.

Taken together, the results summarized in Figure 16.5 reveal several important points. First, the key decisions that influence the outcome of PrP biogenesis (with respect to topology) are made during the synthesis of PrP (i.e., cotranslationally). Second, each step is influenced substantially by interactions between the translocon and elements in PrP (the signal sequence and TMD). Third, these interactions appear to occur with only moderate fidelity, a feature that is critical to the generation of topologic heterogeneity. And fourth, the strength of these interactions can be changed by mutations in the signal or TMD to influence the outcome of PrP topogenesis in predictable ways. These insights not only provide a framework for understanding PrP topogenesis, but facilitate the focusing of subsequent studies on the most important mechanistic steps of potential relevance to disease pathogenesis.

In the case of <sup>Ctm</sup>PrP and cyPrP, the critical step is now revealed to be the signal sequence-mediated translocation of the N-terminus into the ER lumen. The degree of inefficiency at this step determines the percent of nascent PrP chains that have the opportunity to be made as <sup>Ctm</sup>PrP and/or cyPrP. Detailed analysis of this step has demonstrated that it is surprisingly complex and involves several factors (Figure 16.6). First, it is clear that signal sequences from different proteins carry out

**Figure 16.6.** A key branch point in the biogenesis of the different topologic forms of PrP. Nascent PrP polypeptides at ER translocons can go down two pathways. The first involves an interaction between the PrP signal and components of the translocon to mediate translocation of the N-terminus into the ER lumen. This pathway is facilitated by the Sec61 complex, the TRAP complex, TRAM, and ER luminal chaperones such as PDI. Following this pathway is a prerequisite for the generation of <sup>sec</sup>PrP or <sup>Ntm</sup>PrP, both of which have their N-terminus in the ER 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 <sup>Ctm</sup>PrP or cyPrP. This is the default pathway taken by PrP when the minimal translocon composed only of the Sec61 complex is available. Thus, a combination of features encoded in the nascent chain (e.g., the signal sequence) and accessory components of the translocon (such as TRAP and PDI) determine the amount of potentially cytotoxic forms of PrP (e.g., cyPrP and <sup>Ctm</sup>PrP) that are generated.



this step with markedly different efficiencies<sup>112,113</sup>, with the PrP signal being roughly 'average' in this respect. Second, this step involves interactions between the signal sequence and the central component of the translocation channel, the Sec61 complex<sup>114</sup>. Third, the signal-translocon interaction appears to be influenced by at least two proteins termed TRAM<sup>115,116</sup> and the TRAP complex<sup>117</sup>. Fourth, not all signal sequences require TRAM and TRAP for efficient function; while most (including PrP) require at least one of these two complexes, a very small proportion of signal sequences can function well without either<sup>114–117</sup>. And finally, the availability of ER luminal chaperones appears to influence translocation<sup>118,119</sup>, particularly of PrP (our unpublished observations). Although it is not known which chaperones are most important, crosslinking studies indicate an interaction between the N-terminus of PrP and protein disulfide isomerase (PDI) at early steps during PrP translocation<sup>108</sup>.

Thus, avoiding the generation of <sup>Ctm</sup>PrP and cyPrP requires the collective action of numerous determinants that include the signal sequence, Sec61 complex, TRAP complex, ER luminal chaperones, and potentially yet unidentified factors. Conversely, when PrP is synthesized using proteoliposomes containing only the absolute minimal translocation machinery (composed of the SRP-receptor and Sec61 complex), essentially all of the polypeptides are made as either <sup>Ctm</sup>PrP and cyPrP (ref. 120). One of the few signal sequences capable of utilizing this minimal translocon efficiently comes from the protein prolactin<sup>114–117</sup>. All of this assembled information on the key steps of <sup>Ctm</sup>PrP and cyPrP synthesis now provides useful tools that can be used to modulate generation of these forms *in vivo*. For example, one can envision modulating the activity or expression levels of key factors such as the TRAP complex or PDI to influence PrP topogenesis.

Even simpler, at least initially, would be to modify the PrP signal sequence to alter its activity. Indeed, simply replacing the PrP signal with the prolactin signal substantially increases the efficiency of N-terminal translocation, and consequently, decreases generation of <sup>Ctm</sup>PrP (and as demonstrated in more recent unpublished experiments, cyPrP)<sup>112,113</sup>. Remarkably, this manipulation is so effective that, when assayed *in vitro*, it can totally reverse the increased <sup>Ctm</sup>PrP caused by disease-causing mutation in the TMD<sup>108</sup>. Such a manipulation is very valuable because it now allows the testing of hypotheses relating the ability to generate <sup>Ctm</sup>PrP (or cyPrP) to their proposed roles in heritable and transmissible disease. Importantly, the mature domain of PrP is not changed by such changes; only the relative amounts of its topologic forms. Several important ideas, discussed in earlier sections of this chapter, can and should be tested.

First, can the neurodegenerative phenotype of an otherwise disease-causing mutation (such as A117V) be pre-emptively avoided by increasing the efficiency of signal sequence-mediated translocation of the N-terminus? That is, is the neurodegeneration associated with the A117V mutation due to increased  $\text{C}^{\text{tm}}\text{PrP}$  generation, as is currently hypothesized, or to some other effect of this mutation? Second, does reducing generation of cyPrP during its translocation completely preclude generation of cyPrP *in vivo*? Here, the question is whether the majority of cyPrP is generated due to inefficient translocation, as has been suggested, or due to inefficient maturation in the ER followed by retrotranslocation? By eliminating one potential source (due to translocation), the contribution of the other potential source (due to retrotranslocation) can be isolated. Such an experiment will also allow the testing of a related hypothesis: do disease-associated mutations (such as D178N) result in increased retrotranslocation from the ER due to less efficient maturation? And finally, does reducing the propensity to generate  $\text{C}^{\text{tm}}\text{PrP}$  or cyPrP reduce susceptibility to neurodegeneration upon accumulation of prions and  $\text{PrP}^{\text{Sc}}$ ? This is a key prediction of both the  $\text{C}^{\text{tm}}\text{PrP}$  and cyPrP frameworks, and can now be tested.

In general, testing each of these ideas will require relatively long term experiments involving the generation of suitable transgenic mice. However, a relatively high degree of confidence in the productiveness of such an approach is warranted by the *in vitro* analysis suggesting that the manipulations are selective and make specific predictions. In the case of cyPrP, where cell culture assays for its generation and accumulation (in the presence of proteasome inhibitors) have been developed, some of these hypotheses can also be examined in culture. Here, the results are striking. Simply increasing the efficiency of N-terminal translocation nearly completely eliminates cyPrP generation, even under conditions of prolonged proteasome inhibition (our unpublished results). This suggests that in cultured cells under normal conditions, little if any cyPrP is generated by the ER misfolding and retrotranslocation pathway. The consequence of avoiding cyPrP generation is increased resistance to PrP aggregate formation during proteasome inhibition and an accompanying resistance to cell death (our unpublished results). This illustrates the utility and power of a quantitative cell biological approach to understanding otherwise subtle, but potentially important aspects of PrP metabolism. It will now be of great interest to learn the results of currently ongoing transgenic mice studies in which the generation of  $\text{C}^{\text{tm}}\text{PrP}$  and cyPrP have been modulated. Will such manipulations influence the neurodegenerative phase of transmissible prion disease, and if so, how?

As more mechanistic insight is gained into each of the many other steps of PrP biosynthesis and metabolism, yet additional hypotheses

and tools will be generated. These insights should be useful not only for the understanding of PrP biology and the diseases with which it is associated, but also for uncovering novel cell biological principles. In analogous fashion, another idea with roots in PrP 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<sup>121,122</sup>. In fact, although not emphasized in this chapter, the studies on PrP biogenesis have helped identify functions for novel factors in protein translocation (e.g., the TRAP complex)<sup>117</sup>, revealed the complexity and heterogeneity of signal sequences<sup>112,113</sup>, and identified the protein translocon as a potential site for cellular regulation<sup>123</sup>. In the broader sense, the apparently unusual features of PrP (such as its ability to be made in multiple forms) may be a far more general but unappreciated area of cell biology.

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