ACUTE CELLULAR UPTAKE OF ABNORMAL PRION PROTEIN IS CELL TYPE AND SCRAPIE STRAIN INDEPENDENT

Christopher Scott Greil

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ACUTE CELLULAR UPTAKE OF ABNORMAL PRION PROTEIN IS CELL TYPE AND SCRAPIE STRAIN INDEPENDENT

By

Christopher Scott Greil

B.S. Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, 2003

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Approved by:

Dr. David A. Strobel, Dean
Graduate School

Dr. Suzette A. Priola, PhD
Laboratory for Persistent and Viral Disease
Rocky Mountain Laboratory NIH/NIAID

David Poulsen, PhD
Biomedical and Pharmaceutical Sciences

Leonard Evans, PhD
Laboratory for Persistent and Viral Disease
Rocky Mountain Laboratory NIH/NIAID

Keith Parker, PhD
Biomedical and Pharmaceutical Sciences

Richard Bridges, PhD
Biomedical and Pharmaceutical Sciences
Acute cellular uptake of abnormal prion protein is cell type and scrapie strain independent.

Chairperson: Suzette A. Priola

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease, bovine spongiform encephalopathy and sheep scrapie. TSE disease pathology and mechanisms within the central nervous system (CNS) of an infected host largely remains unclear. At the cellular level, the uptake of protease resistant prion protein (PrP-res), which strongly correlates with infectivity and is a valid marker for TSE infection, is one of the earliest events that must occur during TSE infection. Given the difficulty of clearly distinguishing input PrP-res from either PrP-res or protease-sensitive PrP (PrP-sen) made by the cell, the uptake of PrP-res from an infectious inoculum into the host cell remains a poorly understood process. Through the development of a novel assay to exclusively detect input PrP-res we hypothesized that the acute infection of cells by PrP-res is mediated through general processes such as endocytosis, whereas internalization, retention, and propagation of PrP-res are dictated by specific characteristics of both the host cell and PrP-res. Using PrP-res tagged with a unique epitope to the mouse monoclonal antibody 3F4, we developed a detection system to specifically follow the acute cellular uptake of PrP-res. Mouse neural and fibroblast cells were exposed to three different mouse scrapie strains and PrP-res from the inoculum monitored. For all strains, PrP-res uptake was rapid and independent of both cellular prion protein expression and cell type. However, only 30%-40% of the cells were able to internalize PrP-res and PrP-res aggregate size influenced PrP-res uptake. Furthermore, infectious brain homogenate PrP-res was taken up more efficiently then PrP-res in either microsome or partially purified preparations. Our results suggest that PrP-res aggregate size, the PrP-res microenvironment, and/or host cell-specific factors can all influence whether or not a cell takes up PrP-res following exposure to TSE infectivity.
ACKNOWLEDGEMENTS

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INTRODUCTION

1.1 Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs) are a unique group of uniformly fatal neurodegenerative diseases that include kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler-Scheinker syndrome in humans as well as bovine spongiform encephalopathy, sheep scrapie, and chronic wasting disease (CWD) in deer and elk (56). These diseases are often characterized by a long asymptomatic phase, followed by a relatively short clinical phase which progresses to death. The asymptomatic phase of TSE disease can last from a period of months to several years. This long asymptomatic incubation phase of TSE disease has lead to the disease’s historical classification as a slow and persistent viral disease. When the disease reaches clinical stages, victims are subject to a rapid degradation of a variety of neurological functions resulting in memory loss, dementia, myoclonus, ataxia and eventually death (45).

Pathologically many TSEs are characterized by the formation of vacuoles within the brain. The presence of these vacuoles is referred to as spongiosis and their origins remain unclear. TSE diseases are also often characterized by the presence and deposition of polymeric amyloid plaques. Postmortem, the presence of either the spongiosis or amyloid plaques help to identify TSE related illness.

One of the most interesting aspects of TSE disease is that no bacteria or virus has ever been found to be the agent of infectivity. TSE infectivity can remain in tissue which
has been subjected to treatment that would destroy viruses and bacteria. This has led to the hypothesis that the infectious agent in TSE diseases may be a self-replicating protein (1, 32). In the early 1980’s a protease resistant protein was found that strongly associated with TSE infectivity. This protein was termed the prion protein or PrP-res (10).

1.2 The Prion

PrP-res, because of its strong association with TSE infectivity, is considered by many to be the sole component of the transmissible infectious agent of TSE infections (57). PrP-res was determined to be an abnormal isoform of the normal cellular protein PrP (9, 42, 29). The normal form of PrP, which is susceptible to protease digestion, is referred to as PrP-sen. PrP-sen is a ubiquitous glycosylphosphatidylinositol (GPI) anchored protein found in most tissues but with greatest abundance in the central nervous system (65).

The generation and life cycle of PrP in cells is a fairly well understood process (Fig. 1). PrP-sen is translocated into the lumen of the ER where the N-terminal signal peptide is cleaved and a GPI-anchor is attached (35, 9). PrP is then translocated into the Golgi apparatus where as it moves from the cis to trans phase it is taken through the addition and the removal of high mannose sugars at two N-linked glycosylation sites and eventually forms complex glycans (22). PrP then translocates to the cell surface where it resides on the outer membrane of the cell attached to the cell surface via its GPI anchor (73, 22, 74) (Fig. 1). The half life of PrP has been determined to be from 3 – 6 hrs post exposure to the cell surface (23).
PrP can exist in three different glycosylated states: unglycosylated, monoglycosylated, or diglycosylated (Fig. 2). When visualized by Western blot, PrP therefore migrates in three bands representing each glycoform from about 25 – 40 kds. There are distinguishable aspects of secondary structure in PrP that are of note. The N-terminal region of the protein PrP is a long flexible chain of amino acids containing a series of eight, amino acid repeats that have a high affinity for copper ions. This region is identified as the octa-repeat region (Fig. 2). More central in the PrP sequence are two major beta strands (β1 and β2) and three major alpha helical regions (α1-α3) (Fig. 2). These regions are part of the external folded structural domain of PrP. At the C-terminus is the site where the GPI anchor is added following cleavage of the GPI anchor signal site at amino acid residue 232. The GPI anchor tethers PrP to the cell surface membrane (Fig. 1, Fig. 2). Despite many studies and the generation of PrP-sen knockout mice (76, 19, 46), the normal function of PrP-sen remains largely unclear. Mice deficient for PrP do exhibit select learning and memory acquisition deficits as well as some sleep pattern irregularities, although the deficiencies are minor (46).

PrP-res is posttranslationally derived from the normal form of PrP-sen (12, 23) (Fig. 1). PrP-res is distinguishable from PrP-sen in that it is insoluble and resistant to protease degradation (12, 21, 63) and the half life of PrP-res is more then 48 hrs (12, 23). The post-translational, conformationally driven conversion of PrP-sen to PrP-res is a critical, though poorly understood event during TSE pathogenesis (Fig. 3). It is thought that PrP-sen most efficiently converts to PrP-res while both proteins are bound to the same fluid mosaic membrane (4). This sterio-specific positioning of PrP-sen and PrP-res may help to explain the ordered aggregation of PrP-res into amyloid fibrils. It is thought that new
PrP-sen is converted to PrP-res when it is added to the ends of growing PrP-res aggregate fibrils in a stacking or helical fashion (Fig. 3). Smaller PrP-res fibrils have been shown to be more infectious than larger PrP-res fibrils (71). This suggests that smaller PrP-res fibrils contain a greater abundance of active conversion sites per unit volume of PrP-res aggregate than volumes larger PrP-res fibrils. As PrP-sen converts to PrP-res its secondary structure radically changes from a highly soluble protein of predominately alpha helical character to an extremely stable and insoluble highly beta sheet character (61), a process which likely expands the pool of transmissible agent aiding in the progression of disease (Fig. 2). The differences in secondary structure between PrP-sen and PrP-res likely account for the observed biophysical differences between the two molecules. The entire process of conversion of PrP-sen to PrP-res is thought to occur at either the plasma membrane, in the extracellular space or along the endocytoic pathway (23, 48) (Fig. 1). In vivo, PrP-sen to PrP-res conversion can lead to PrP-res aggregation and potential persistence in the extracellular space or cell surface as amyloid fibrils or in select endocytoic compartments (Fig. 1) (39, 25). However, in vitro, the propagation of infectious PrP-res by PrP-sen conversion has been shown to be driven simply with the kinetic energy of agitation and catalytic application of polyanionic compounds (27).

The spread of PrP-res and disease progression throughout the CNS appears to occur in a multidirectional fashion along nerves at the site of infection (52). With respect to scrapie disease, regardless of the site of initial nerve infection, PrP-res is generated in the CNS and can travel from the CNS back out to peripheral nerves. The site of infection can, however, alter disease incubation times. For example, direct injection into the CNS (via intracerebral inoculation) generates clinical symptoms more quickly than inoculation
via the oral route while intraspinal injections are more effective at shortening disease incubation times then intacerebral injection (41).

As PrP-res is membrane bound via a GPI anchor, spread of disease through the CNS is thought to occur via the transport of infected vesicles (3). This argument is reinforced by findings from mice that express PrP without a GPI anchor. When these mice are infected with scrapie, they produce large amounts of PrP-res yet develop no clinical disease (25). The spread and deposition of non GPI anchored PrP-res is also different from infected wild-type mice (25). The GPI anchorless PrP mice deposit PrP-res in large plaques that only exist in the extracellular spaces, while wild-type mice can exhibit much more diffuse PrP-res deposition. These findings reinforce the notion that the GPI anchoring of PrP is important in disease propagation and clinical development.

1.3 Strains

The transmission and propagation patterns of TSE disease in vivo seem to be dictated by both the genetic profile of the host and select characteristics of the TSE agent. TSE agents do exhibit strain variation (17). TSE strains are defined both biochemically and pathologically. Biochemically, strains are defined by the predominant glycosylation pattern they display and the size of their PrP-res molecules. Pathologically, different strains are defined by reproducible pathology observed when the agent is serially passaged in genetically similar animals. This repetitive pathology is characterized by disease incubation time, distribution of PrP-res in the brains, distribution of spongiosis change, and neural loss (18). Factors which ultimately dictate strain type remain unclear, although studies have shown that strain type is not dictated by the primary amino acid
Figure 1: Life cycle of PrP and the propagation of PrP-res
The maturation and expression of PrP-sen (PrP\textsuperscript{C}) is as follows: 1) PrP-sen (yellow circles) is translated into the lumen of the ER and is tethered via a GPI anchor to vesicular wall. 2) PrP-sen is transported to the Golgi where it is glycosylated through a high mannose step and is then reduced to form complex glycans. 3) PrP-sen is transported from the trans face of the Golgi to the cell surface. The cellular conversion and propagation of PrP-sen to PrP-res (PrP\textsuperscript{sc}, orange barrels) is thought to occur at the following: 4) Cell conversion of PrP-sen to PrP-res can occur at the cell surface (a), or in an endosome / lysosome compartment (b). 5) The propagation of PrP-res can ultimately lead to PrP-res aggregate in the form of extracellular amyloid plaque (a), cell surface aggregates (b), or aggregates accumulating within endosomal / lysosomal compartments (c) (Figure adapted from ref # 55) (\textsuperscript{55}).
Figure 2: A graphical representation of PrP. This display highlights the major structural features of the PrP amino acid sequence. One should note the following features: 1) The signal peptide cleavage site, 2) The octa-repeat region which consists of an eight amino acid sequence repeated 5 times, 2) The locations of the 3F4 and L42 epitopes 3) The two major beta strands (β1 and β2) and three major alpha helices (α1-α3), 4) The two N-linked glycosylation sites at asparagines 181 and 197, 5) The GPI anchor addition on PrP.
Figure 3: Conversion and propagation of PrP-res from PrP-sen
PrP-sen (blue circles) is converted to PrP-res (blue squares) after binding to PrP-res (red squares) a process that is self propagating. The conversion from PrP-sen to PrP-res is irreversible and expands the PrP-res pool. As PrP-res is propagated it aggregates and can order to form amyloid fibrils. These fibrils propagate in length by the further conversion of PrP-sen to PrP-res at the ends.
sequence of PrP as multiple strains have been defined in mice expressing the same PrP genotype \cite{16}. Many studies suggest that strain determination is strongly controlled by the inoculum PrP-res and that each TSE strain represents a specific inoculum PrP-res conformation \cite{28, 64}. However, other studies have shown that host animal genotype or species can influence disease susceptibility via specie barriers clinical symptoms and PrP-res propagation \cite{51}.

At the cellular level, the respective interactive roles host cells and exogenous TSE agent play in determining TSE strain type remain unclear, although strain type may influence infection via PrP-res binding to cells, uptake into cells, or trafficking and localization in cells. These critical steps outlined in Figure 1, may be impacted by both the cell type and the strain of TSE. Previous work has suggested that scrapie strain type may influence whether or not a particular cell type can become chronically infected with scrapie as only certain scrapie strains can chronically infect select cell types \cite{79}.

1.4 Cellular dynamics of TSE infection

Certain cell types when exposed to scrapie infected brain homogenate will produce PrP-res over multiple serial passes, and when injected into experimental animals, cause disease. These cells are considered to be persistently infected. A number of studies have shown that both neuronal and non-neuronal cell lines can become persistently infected with certain strains of mouse scrapie, sheep scrapie or CWD \cite{3, 20, 58, 60, 62, 68, 79, 80}. The vast majority of what is understood about the process of cell infection and PrP-res formation has been the result of studies on such persistently infected cells. These studies have shown that PrP-res formation in infected cells occurs
at the cell surface and/or along the endocytic pathway (6, 14, 23) and that cell associated PrP-res has a long half life (13, 23) and can accumulate in endolysosomal compartments (24, 48, 54, 75). Although cells persistently infected with scrapie agent have provided important insights into the cellular biology of PrP-res formation during infection, very little is known about how PrP-res is initially taken up by cells.

Several recent studies have looked at the earlier stages of TSE infection with some data suggesting that PrP-res is taken up rapidly (i.e. within 24 hrs) (34, 36, 50, 53) and other data indicating that uptake may occur more slowly (i.e. over days) (7, 43). Most of these earlier studies were limited by the difficulty of specifically distinguishing PrP-res in the inoculum from PrP-res newly made in the cells which may help to explain the conflicting results. Even in instances where partially purified PrP-res was uniquely labeled, it was not always possible to absolutely distinguish the labeled PrP-res from other labeled, but non-relevant, protein contaminants (43). Additionally, few studies have examined more then one strain of TSE agent meaning that any potential differences in strain-specific PrP-res uptake early during infection remain largely unresolved.

Using uniquely epitope tagged PrP-sen molecules it has previously been shown that, regardless of scrapie strain, both mouse neuroblastoma (MNB) cells as well as mouse fibroblast cells form de novo PrP-res after only 4 hrs of scrapie exposure (79). This finding strongly suggests that the association of PrP-res with a cell occurs rapidly. However, it remains unclear, how and with what compartments input PrP-res interacts with host cells. Data from the same study shows that when acutely infected cells are passaged several times only certain host cell and scrapie strain combinations can become
persistently infected (79). This finding raises questions as to whether or not the initial interactions of different scrapie strains with host cells are similar or different.

Without the definitive ability to examine input PrP-res independent of PrP-sen or host converted PrP-res a number of questions remain unanswered in regards to the acute infection of cells (0 – 72 hrs) via inoculum PrP-res. These questions are: 1) Do input PrP-res molecules become primarily internalized by the host cell? 2) Is PrP-res uptake a rapid process? 3) Do different cell types or the expression of PrP-sen alter PrP-res uptake? 4) How is PrP-res taken up into cells? 5) Does PrP-res uptake reflect strain differences? 6) Does altering the preparation of PrP-res affect its uptake into cells?
1.5 Overall Hypothesis and Aims

In light of the unanswered questions raised in the introduction, we undertook an *in vitro* study to examine how different strains of inoculum PrP-res interact with different cells during the acute stage of scrapie infection. Based upon previous work we hypothesized that the acute infection of cells by PrP-res is mediated through general processes such as endocytosis, whereas internalization, retention, and propagation of PrP-res are dictated by specific characteristics of both the host cell and PrP-res. To address this hypothesis we developed a novel method for selectively detecting inoculum PrP-res independent of PrP-sen or converted PrP-res. With this new technique we conducted experiments to address the above listed questions. To test our hypothesis the aims of the study were as follows:

1.) Determine if inoculum PrP-res could be detected independent of PrP-sen or newly formed host derived PrP-res.
   - Ensure select PrP-res is uniquely detectable via Western blot bioassay.
   - Develop a select PrP-res detection system via fluorescent microscopy.

2.) Evaluate the basic kinetics of inoculum PrP-res uptake into cells during acute infection.
   - Determine PrP-res uptake.
   - Determine if different cell types effect PrP-res uptake.
• Determine if host cell derived PrP-sen aids in PrP-res uptake.
• Evaluate the cellular distribution of PrP-res.

3.) Determine by what process PrP-res is taken up by cells.
• Determine if PrP-res uptake is an active cellular process.
• Determine how PrP-res might be trafficked in cells.

4.) Determine how different PrP-res strains and purification techniques may alter uptake into cells.
• Assess if different PrP-res strains contain unique biophysical characteristics.
• Evaluate how PrP-res aggregate size may influence uptake.
• Assess how different inoculum PrP-res preparations might alter cellular uptake.
Chapter 2
MATERIALS AND METHODS

2.1 PrP<sup>0/0</sup> Cell Lines (developed by Dr. Ina Vorberg)

Pregnant mice in which the PRNP gene has been knocked out (PrP<sup>0/0</sup>) (46) were anesthetized with isofluorane at gestation day 15. Embryos were isolated, decapitated and the heads were transferred to cold dissecting solution (phosphate buffered saline (PBS), Ca<sup>2+</sup>/Mg<sup>2+</sup> free, 5% glucose). Brains were removed and freed of meninges and blood vessels, cut into 5 mm<sup>2</sup> pieces, and disassociated by aspiration in Dulbecco’s phosphate buffered saline (DPBS) with 10% FBS (Gibco Inc.), 0.5 mM glutamine, and B27. The cell suspension was centrifuged at 160 X g, 4°C, 5 min and cells were resuspended in DMEM with 5% FBS, and F12. Cells were adjusted such that 40,000 cells/well were plated on poly-L-lysine coated 24 well plates. After 4 hrs incubation at 37°C, 5% CO<sub>2</sub>, medium was replaced with 1 ml/well of DMEM with growth supplements FBS (3%), B27, and N2. Transfection of cells for immortalization was done 48 hrs after plating, using the plasmid vector pSV3-neo (ATCC, #37150) (72) and lipofectamine (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, G418 was added to the medium to select for transfected, immortalized cells. Immortalized clones were pooled and single cell clones were established. A single cell clone (CF10) was selected and maintained in Opti-MEM supplemented with 10% FBS and 1% penicillin streptomycin (PS). CF10 cells are strongly positive for nestin (data not shown) which is predominantly found in stem cells of the CNS. Therefore, it is likely that they are neural stem cells.
MoL42-CFD5 and Mo3F4-CF10 cell lines were derived by limiting dilution cloning from CF10 cells that had been transduced with a retrovirus encoding the mutant PrP molecule of interest (44). The Mo3F4-CF10 cells express mouse PrP with the epitope to the mouse monoclonal antibody 3F4 (Mo3F4) (Fig. 3), while the MoL42-CFD5 cells express mouse PrP with the epitope to the mouse monoclonal antibody L42 (MoL42) (see Fig. 2 for epitope location) (77). The L42 epitope can be used to distinguish mouse PrP in the cells from mouse PrP in brain homogenate.

2.2 Fibroblast Cell Lines

Fibroblast cells (ψ2) are susceptible to infection with the mouse scrapie strain 22L but not with the ME7 strain (79). MoL42-ψ2A2 is a cell line derived by limiting dilution cloning from ψ2 fibroblast cells (44,49) that had been transduced by the same retrovirus used to make the MoL42-CFD5 cells and Mo3F4-CF10 cells. MoL42-ψ2A2 cells express both endogenous mouse PrP as well as mouse PrP with the L42 epitope. MoL42-ψ2A2 cells were maintained in RPMI with 10% FBS supplemented with 1% penicillin/streptomycin (final concentration of 100 units/ml penicillin G and 100 ug/ml streptomycin) (Invitrogen). Ha3F4-ψ2A6 cells are a hamster derived fibroblast cell line which express hamster PrP while naturally expressing the 3F4 epitope. Ha3F4-ψ2A6 cells were maintained in DMEM with 10% FBS supplemented with 1% PS. The L929-Mo3F4 cell line was a mouse L929 fibroblast cell line derived from limited dilution cloning which had been transduced with a retrovirus (44) to express mouse PrP with the 3F4 epitope. L929-Mo3F4-22L cells were L929-Mo3F4 cells which had been
persistently infected with mouse scrapie strain 22L (80). Approximately 47% of these cells express high levels of 3F4 positive PrP-res by western blot (80).

2.3 Cholinergic Septal Neural Cell line

SN56 cells are neuronal derived cells from mouse septum neurons (33) and were used with the kind permission of Dr. Bruce Wainer (Department of Pathology, Emory University of Medicine, Atlanta, GA). The SN56 cells were maintained in Opti-MEM and differentiated with 1mM dibutyryl cyclic AMP as previously described (2, 8). A listing of all cell types and the epitope-tagged PrP molecule which they express can be found in Table 1. With the exception of the CF10 cells, all cell types express endogenous, non-epitope tagged wild type PrP.

2.4 PrP-res preparation

Scrapie strains 22L and ME7 were the kind gift of Dr. James Hope (Vitechnologies, Boston, Ma) while the Obihiro scrapie strain (70) was the kind gift of Dr. Motohiro Horiuchi University of Obihiro, Hokkaido, Japan). Tg(WT-E1) mice were from the laboratory of Dr. David Harris (Washington University, St. Louis, Mo). These mice express high levels of PrP-sen with an epitope tag (11) for the mouse monoclonal antibody 3F4 (PrP-sen3F4) (Fig. 2).

The mouse-adapted scrapie strains 22L, ME7, and Obihiro (Obi) in DPBS were passaged once in Tg(WT-E1) mice (26) to generate the brain homogenates 22L(3F4), ME7(3F4), and Obi(3F4). Tg(Wt-E1) mice inoculated with uninfected C57Bl/10 mouse brains were designated as mock controls (Mock(3F4)). Brains from terminally ill
<table>
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<th>PrP expression</th>
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<td>CF10</td>
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<td>N/A&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>SN56</td>
<td>Cholinergic&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mouse</td>
<td>Yes</td>
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</table>

**Table 1: Cell lines used.** With the exception of the CF10 cells, all cell types also express endogenous, non-epitope tagged wild-type PrP.

<sup>a</sup>: N/A = not applicable

<sup>b</sup>: neural differentiated
Tg(WT-E1) mice or mock infected, age matched controls were harvested and stored at -70°C. To study the uptake of PrP-res from crude brain homogenate, brains were dounce homogenized in DPBS (10% w/v), sonicated, and then stored at -70°C as described previously (79). Crude brain microsome fractions were prepared as described in detail by Baron and colleagues (4), while PrP-res was partially purified as previously described (59) but without proteinase K (PK) digestion. Partially purified PrP-res was quantified by western blot using a standard protein concentration curve derived from recombinant hamster PrP-sen. For experiments where partially purified PrP-res preparations were compared to less purified microsome or brain homogenate preparations, total protein content was normalized using microsomes or brain homogenate from mock-infected Tg(WT-E1) mice. Alexa-Fluor-596 labeled PrP-res preparations were made as previously described by Magalhães and colleagues (43).

2.5 Cellular uptake of PrP-res

To analyze PrP-res uptake over time, experiments were performed in 24 well microtiter plates as previously described (79). Briefly, different cell numbers were initially plated in order to ensure similar cell numbers at time of harvest. The number of cells plated were: MoL42-CFD5 cells (0-24 hrs, 3 X 10^5 cells/ml; 48hrs 2 X 10^5 cells/ml; and 72 hrs 1.5 X 10^5 cells/ml), CF10 and MoL42-ψ2A2 cells (0-24 hrs 4 X 10^5 cells/ml; 48hrs 2 X 10^5 cells/ml; and 72 hrs 1.5 X 10^5 cells/ml). After incubation at 37°C for 24 hrs, the medium was removed and replaced with 200 μl of brain homogenate, microsomes, or partially purified PrP-res diluted in Opti-MEM. The cells were exposed to equivalent amounts of PrP-res for each strain (10 ng for each preparation). Mock
preparations were diluted 1:10 in Opti-MEM. Samples were then incubated at 37°C for
2-4 hrs followed by the addition of 400 µl/well of fresh media. At each time point, cells
were washed 4 times with 300 µl of fresh medium and either removed from the plate
using trypsin-EDTA (Invitrogen) and counted with a hemacytometer or lysed directly in
3X lysis buffer (3 mM Tris-HCl, pH 7.4, 420 mM NaCl, 15 mM EDTA, 1.5% sodium
deoxycholate, and 1.5% Triton X-100). Lysates were treated with benzonase (0.167
U/ul) (Novagen) for 30 min at 37°C, then PK (60 µg/ml) (Roche) for 1 hr at 37°C
followed by the addition of 1.2 µg of 4-(2-aminoethyl)benzenesulfonylfluoride
(PEFABLOC) (Roche). PK digested lysates were precipitated in four volumes of cold
methanol for 2 hrs at -20°C followed by centrifugation at 20,800 X g for 30 min. Pellets
were sonicated into sample buffer (2.5% SDS, 3 mM EDTA, 2% β-mercaptoethanol, 5%
glycerol, 0.02% bromphenol blue, and 63 mM Tris-HCL, pH 6.8), boiled for 3 min,
optionally PNGaseF treated for 12 hours according to the manufacturer’s instructions
(New England Biolabs), and loaded on 16% Tris-Glycine precast gels (Invitrogen). PrP
was detected by western blot analysis using the mouse monoclonal antibody 3F4
(1:3,000) followed by secondary ECL-anti-mouse IgG (1:5,000) (Amersham) or anti-
mouse IR-dye 800CW (1:10,000) (Li-Cor) (Table: 2). Quantitative ECL data was
generated using the UN-SCAN-IT software (Silk Scientific Corp.) according to the
manufacturer’s instructions. In order to quantify the data within the linear range of the
film, the first three lanes of each gel were used as internal standards to establish a
constant film exposure time which was then used for every experiment. PrP-res levels
were quantified from gels exposed to film for a set time (4 min) using a fixed parameter
box (i.e. the box was the same volume for every experiment) and the UN-SCAN-IT
software. Pixel count totals within the fixed parameter box were summed and presented as pixels. When the secondary antibody anti-mouse IR-dye 800CW was used to develop the western blot, quantitative data were obtained using the Li-Cor Odyssey IR scanner and the software provided with the system (Li-Cor).

2.6 Detection of PrP-sen by fluorescent microscopy

Cells expressing mouse PrP-sen with either the 3F4 or monoclonal antibody L42 epitopes were plated in Lab-Tek II chamber slides (8-well) (Nalge Nunc Inc.) and grown to approximately 70% density. Cells were then fixed in 200µl of 10% formaldehyde for 30 min, followed by 200 µl of 0.4% triton-X-100 for 10 min. The wells were then rinsed with 500 µl of PBS (2X). Cells expressing mouse PrP with the L42 epitope were hydrolytically autoclaved in Citrate buffer (0.18 mM citric acid and 0.82 mM Sodium citrate dissolved in distilled water, pH 6) at 120°C for 8 minutes and then cooled. 200 µl of the mouse 3F4 monoclonal antibody conjugated to TRITC dye (1:200 dilution) or mouse L42 monoclonal antibody (1:200 dilution) was added for 30 min and followed by PBS washes (3X) (Table 2). L42 treated cells were then treated with of 200 µl of goat anti-mouse FITC antibody (1:400 dilution) for 30 min. Following the antibody incubations, the slides were rinsed in PBS and coverslip mounted with Mowiol 4-88 (Calbiochem). Slides were viewed on a Nikon Microphort-SA fluorescent microscope with a 40X air immersion objectives. Images were captured on an Olympus Q-Fire monochrome camera and color designated with Adobe Photoshop.
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**Table 2:** Antibodies list used for experiments. The antibodies are listed by species, epitope, and the appropriate dilution for use by either Western blot<sup>a</sup> or immunofluorescent assay<sup>b</sup>.  

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2.7 Development of assay to detect inoculum PrP-res via immunofluorescent microscopy

Developing an assay to detect the inoculum PrP-res which had been introduced to the host cells was one of the major objectives of this study. Using established techniques for the detection of PrP-res in infected tissue (37, 38) as a starting point, we worked to develop a similar protocol which could be used on to a cell monolayer. Regardless of tissue preparation method, all of the published protocol followed the same general format. Tissue was first fixed and then treated with detergent in order to permeabilize the cell. The tissue could then be treated with PK to remove PrP-sen if necessary and subsequent anti-PrP antibodies to detect PrP-res.

We had determined from a previous Western blot analysis that non-cell associated PrP-res could be removed from cells after several PBS rinses (Fig. 9). However, the rinsing of cell monolayers prior to fixing revealed that live cells actually retract when subjected to the PBS rinses shrinking the cells and making evaluation of the fluorescent signal more difficult. Washing the cells with warm media corrected the problem. A variety of formaldehyde treatments were tested (Table 3) and we determined that fixing cells in 3.7% formaldehyde was sufficient to fix cells while maintaining cell morphology. Cold MeOH was also evaluated, but it destroyed the cellular morphology making it impossible to interpret the results. Permeabilization was accomplished with Triton-X-100 and different concentrations were evaluated to establish an optimal of 0.4% (Table 3). We found that when attempting to detect PrP-res in cells that also expressed PrP-sen it was necessary to remove the PrP-sen in order to isolate the PrP-res. We utilized PK in these situations to remove the PrP-sen. The difficulty in treating the cells was that, if left
on too long or in too high of a concentration, the PK would loosen the cells from the slides and the majority of the cells would be lost. Careful testing of a range of PK concentrations and digestion times revealed that a 9 minute exposure of 10 ug/ml of PK would remove the PrP-sen in most cells without removing the cells from the slide.

Due to the fact that the 3F4 epitope is buried in PrP-res it was necessary to denature the protein in order to expose this epitope for detection. We initially tested formic acid for this purpose and found that it generated staining that could be seen regardless as to whether or not the cells were exposed to inoculum PrP-res\textsuperscript{3F4}. The use of guanidine thiocyanate remedied this problem and positive signal was found to be specific to PrP-res. We found that 2M guanidine thiocyanate added to fixed, infected cell monolayers for 30 minutes in order to denature PrP-res\textsuperscript{3F4} was sufficiently to detect PrP-res\textsuperscript{3F4}. Once this step was completed, the mouse antibody 3F4 was used as a primary antibody (1:200) while a fluorescent secondary antibody was used to visualize PrP-res with immunofluorescent microscopy. The different parameters tested and the optimized protocol used are summarized in Table 3.

2.8 Validation of PrP-res\textsuperscript{3F4} detection in cells by immunofluorescent microscopy

The protocol explained in Table 3 was tested on 22L scrapie infected cells persistently expressing mouse PrP-res\textsuperscript{3F4} (Fig 4). PrP-res\textsuperscript{3F4} was detected in the infected cells (Fig. 4B), while the uninfected cells were negative for PrP-res (Fig. 4A). Thus, the assay only detects PrP-res and not PrP-sen. To further validate the assay, previous sub-cloning of cells had determined that approximately 60% of the cells contained PrP-res (data not shown). This was consistent with our data which showed that approximately
<table>
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**Table 3: Developed techniques for detection of PrP-res\textsuperscript{3F4} via immunofluorescent microscopy.** This table outlines the variety of parameters tested in developing an efficient method for detecting PrP-res in exposed host cells by immunofluorescent microscopy. All figures in bold text represent the parameters used in the final protocol.
60% of the infected cells had PrP-res$^{3F4}$ (Fig 4). In order to see if our protocol could be used to detect exogenous PrP-res$^{3F4}$ added to uninfected cells we added PrP-res$^{3F4}$ to mouse neural cells. Cells exposed to exogenous PrP-res$^{3F4}$ did display PrP-res$^{3F4}$ and it was easily distinguishable from inoculum PrP-sen (Fig. 5A). The distribution of PrP-res$^{3F4}$ detected in infected cells remained the same regardless as to whether the inoculum was treated with PK prior be being added to the cells (Fig. 5C,D). These results suggest the assay was specific for PrP-res$^{3F4}$ detection.

2.9 Analysis of PrP-res uptake by fluorescent microscopy

Cells were plated in Lab-Tek II chamber slides (8-well) (Nalge Nunc Inc.) at the densities listed in section 2.5. After 24 hrs at 37°C, the media was removed and immediately replaced with 200 µl of Opti-MEM containing 3 ng of partially purified PrP-res from 22L(3F4), ME7(3F4), or Obi(3F4) or volume matched partially purified PrP-res Mock(3F4) preparations. Following incubation from 2 to 48 hrs, the media was aspirated and the cells were washed 4 times with fresh medium. 200ul of 10% formaldehyde was added to each well for 30 min, followed by 200 µl of 0.4% triton-X-100 for 10 min. The wells were then rinsed with 500 µl of PBS (2X), and select chambers were treated with 200 µl of PK (10ug/ml) for 9 min at 37°C. The PK was then removed and excess PK blocked with 200 µl of PEFABLOC (10mM). Wells were washed again with PBS and treated with guanidine thiocyanate (4M, 200 µl/chamber) for 30 min at 25°C. After rinsing in PBS, 200 µl of the mouse 3F4 monoclonal antibody (1:200 dilution) was added
Figure 4: Detection of PrP-res in chronically infected cells by immunofluorescence. L929-Mo3F4 cells which were either A) uninfected or B) chronically infected with 22L scrapie were rinsed, fixed and immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse FITC labeled antibody (green) was used to detect PrP-res$^{3F4}$ while DAPI stain (blue) denotes cell nuclei. All images were taken with a 40X objective.
Figure 5: Detection of PrP-res by immunofluorescence. MoL42-CFD5 cells were treated for 24 hours with partially purified PrP-res$^{3F4}$ from: A) mock scrapie preparation, B) mock scrapie preparation which was pretreated with PK, C) 22L(3F4) scrapie preparation, or D) 22L(3F4) scrapie preparation which was pretreated with PK. All cells were rinsed, fixed and immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse FITC labeled antibody (green) was used to detect PrP-res$^{3F4}$ while DAPI stain (blue) denotes cell nuclei. All images were taken with a 40X objective.

for 30 min followed by PBS washes (3X) and the addition of 200 µl of goat anti-mouse FITC conjugated antibody (1:400 dilution) for 30 min (Table 2). Following the antibody
incubations, the slides were rinsed in PBS and coverslip mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were viewed on an Olympus BX51 fluorescent microscope with both 20X and 40X air immersion objectives. All images were taken with an Olympus DP71 camera and were processed and analyzed with Microsuite Software (Olympus).

For comparison of PrP-res to Alexa-Fluor-596 conjugated PrP-res, SN56 cells were plated in Lab-Tek II chamber slides (8-well) (Nalge Nunc Inc.) at a 1:20 dilution from a confluent 25 cm² flask and differentiated with cAMP (1mm) in serum free Opti-MEM for 24 hrs. PrP-res or Alexa-Fluor-596 conjugated PrP-res (8 ng) was added to the cells and the cells were incubated for 24 hrs at 37°C. Cells were then fixed, labeled and observed by fluorescent microscopy as described above.

2.10 Endocytosis markers and colocalization techniques by confocal microscopy

CF10 cells were plated in Lab-Tek II chamber coverslip slides (8-well) (Nalge Nunc Inc.) at 1.5 X 10^5 cells/ml. After 24 hrs at 37°C, the media was removed and immediately replaced with 200 µl of Opti-MEM containing 3 ng of partially purified PrP-res from 22L(3F4). Following a set incubation time, the cells were rinsed, fixed and permeabilized as listed above. The cells were then treated with guanidine thiocyanate (2M, 200 µl/chamber) for 30 min at 25°C. After rinsing in PBS, 200 µl of PBS with mouse 3F4 monoclonal antibody (1:200 dilution) and either Alexa-Fluor-596 conjugated cholera toxin subunit B (4 ul/well, Invitrogen), rabbit anti-calnexin (1:300, Sigma), rabbit anti-early endosomal antigen 1 (1:50, Sigma), rabbit anti-LAMP1 (1:50, Sigma), rabbit anti-LAMP2 (1:50, Sigma), or anti-γ-tubulin conjugated to Cy3 dye (1:75, Sigma) were
added for 30 min Table 2. This was followed by PBS washes (3X) and the addition of 200 µl of goat anti-mouse Alexa-Fluor-488 conjugated antibody (1:600 dilution) and in most cases goat anti-rabbit Alexa-Fluor-596 conjugated antibody (1:1000 dilution) for 30 min Table 2. Following the antibody incubations, the slides were rinsed in PBS and 200 ul of PBS with DRAQ-5 (1:250, Alexis) were added to each well. The cover lids were secured to the slides with paraffin wrap for viewing by confocal microscopy. Images were captured with a Zeiss LSM 510 META multi-photon excitation fluorescence microscope, with either a 63X oil immersion or 40X water immersion objective. For all images, the best z-stack distances were established by the provided Zeiss software.

2.11 Sucrose gradient assay

Forty microliters of 10% brain homogenate in DPBS or 40 µl of Prestained SDS-PAGE Standards Low Range (Bio-Rad) were layered on top of a 4.5 ml 10-60% sucrose gradient. Samples were then centrifuged at 232,470 X g in a 100.3 rotor (Beckman) for 16 hrs at 4°C. Ten different 454 µl fractions were carefully collected from the top of the tube using a syringe and canula. Fractions analyzed for PrP-res were treated with 20 ug/ml PK for 1 hr at 37°C followed by the addition of 10 ul (0.1M) PEFABLOC. Following MeOH precipitation, pellets were sonicated into sample buffer, and run on SDS-PAGE for immunoblot analysis as described above.
2.12 PK resistance of PrP-res$^{3F4}$

PrP-res (8ng) from 22L(3F4), ME7(3F4), or Obi(3F4) was diluted in 300 µl of 3X lysis buffer and digested with PK (3 mg to 12 ug) for 1hr at 37°C. Lysates were then treated with PEFABLOC, methanol precipitated, boiled in sample buffer, and run on SDS-PAGE gels for immunoblot analysis as described above.
Chapter 3

RESULTS

3.1 The density average and PK resistance of PrP-res\textsuperscript{3F4} aggregates from 22L(3F4), ME7(3F4), and Obi(3F4) scrapie

With the intent of using scrapie strains 22L(3F4), ME7(3F4) and Obi(3F4) in a variety of different \textit{in vitro} experiments it was necessary to first assess any biophysical differences amongst the strains. Scrapie strains 22L, ME7 and Obihiro possess very distinctive \textit{in vivo} pathologies \cite{40,82,70} and PrP-res glycosylation patterns \cite{78}. Utilizing mouse brain homogenates from scrapie strains 22L(3F4), ME7(3F4) or Obi(3F4) we tested how the different strain’s PrP-res\textsuperscript{3F4} aggregates might differ with respect to average density. Scrapie-infected brain homogenate was applied to a 10 – 60\% sucrose gradient and individual fractions were analyzed for PrP-res\textsuperscript{3F4}. PrP\textsuperscript{3F4} aggregates from uninfected brain homogenates were found primarily in fraction 3, while PrP-res\textsuperscript{3F4} from 22L(3F4), ME7(3F4) and Obi(3F4) were found primarily between fractions 4 and 6 (Fig. 6A). These data suggest that there is little difference between the strains with respect to the average density of PrP-res\textsuperscript{3F4} aggregates.

To determine whether PrP-res\textsuperscript{3F4} from the different scrapie strains might have different susceptibility to PK digestion, equivalent amounts of 22L(3F4), ME7(3F4), and Obi(3F4) were digested with increasing concentrations of PK and analyzed for residual PrP\textsuperscript{3F4}. As shown in Fig. 6B, increasing amounts of PK reduced the pool of PrP-res for each strain in a linear fashion. However, there was no statistically significant difference
Figure 6: Relative density gradient and PK resistance of PrP-res\textsuperscript{3F4} from three mouse scrapie strains. A) Brain homogenate from scrapie strains 22L(3F4), ME7(3F4) and Obi(3F4) were separated by density across a sucrose gradient (10-60\%) into 10 parts and quantified by western blot for PrP-res\textsuperscript{3F4} (N=3) where the error bar represents SEM. No significant difference in density was found between the different scrapie strains (p>.05, Two-way ANOVA). Molecular mass markers are indicated by the boxes. B) Brain homogenates from scrapie strains 22L(3F4), ME7(3F4) and Obi(3F4) were digested with increasing concentrations of PK for 1hr at 37°C and the remaining PrP\textsuperscript{3F4} was quantified by western blot (N=3) where error bars represents SEM. PrP-res\textsuperscript{3F4} from the different strains of scrapie were similarly resistant to PK treatment (p>.05, Two-way ANOVA).
in PK resistance amongst the PrP-res\textsuperscript{3F4} molecules from the three strains. These results suggest that, with respect to average aggregate density and PK susceptibility, PrP-res\textsuperscript{3F4} from all three scrapie strains behaved similarly.

3.2 Characterization of cells and detection of PrP

In order to assess the expression levels of our epitope PrP molecules in the different cell lines, we ran Western blots to detect either L42 or 3F4 epitope-tagged PrP-sen (Fig. 7). Mouse PrP\textsuperscript{0/0} cells (CF10) were negative for PrP and acted as negative controls (Fig. 7). PrP-sen in both the MoL42-CFD5 and MoL42-\psi2A2 cells was detected by the L42, but not the 3F4, monoclonal antibody. Thus, monoclonal antibodies 3F4 and L42 could be used to selectively detect Mo3F4 and MoL42 PrP-sen respectively. Immunofluorescence microscopy was also used to assess the presence of L42 or 3F4 epitope-tagged PrP-sen from select cell lines (Fig 8). PrP expressed by both the Ha3F4-\psi2A6 and MoL42-CFD5 cells were distinguishable from the negative CF10 control cells (Fig 8B, D). Arrows designate the heavy punctate staining of PrP in the Golgi. These results show that epitope-tagged PrP-sen can be specifically detected by both Western blot and immunofluorescence microscopy.

3.3 PrP-res\textsuperscript{3F4} uptake by mouse neural cells

To determine whether or not our mouse neural cells were able to take up PrP-res\textsuperscript{3F4}, MoL42-CFD5 cells were exposed to 22L(3F4), ME7(3F4), Obi(3F4) or uninfected Mock(3F4) brain homogenates. MoL42-CFD5 cells are mouse neuronal PrP\textsuperscript{0/0} cells that have been modified to express mouse PrP-sen that is recognized by the mouse
Figure 7: Expression of epitope-tagged PrP-sen. Western blot detection of PrP-sen with either mouse monoclonal antibody 3F4 (left panel) or mouse monoclonal antibody L42 (right panel) for cell lines CF10 (lanes 1, 5), MoL42-CFD5 (lanes 2, 6), and MoL42-Ψ2A2 (lanes 3, 7). As a positive control for the specificity of the 3F4 antibody, Lane 4 shows CF10 cells expressing Mo3F4.
Figure 8: PrP-sen detection using immunofluorescence microscopy. Cells were rinsed, fixed and immunolabeled for the detection of PrP-sen. CF10 cells (A) or Ha3F4-ψ2A6 cells (B) were immunolabeled with mouse monoclonal antibody 3F4 directly conjugated to TRITC dye (red). CF10 cells (C) or MoL42-CFD5 cells (D) were immunolabeled with mouse monoclonal antibody L42 and secondary anti-mouse FITC labeled antibody (green). Arrows designate heavy PrP deposition in the Golgi.
monoclonal antibody L42 allowing us to distinguish it, if necessary, from mouse PrP in the brain homogenate. MoL42 PrP-sen is not recognized by the 3F4 mouse monoclonal antibody (Fig. 7). After 24 hrs incubation time the homogenate was removed and cells were rinsed to wash off any unbound PrP-res$^{3F4}$, lysed and assayed for PrP-res$^{3F4}$ by Western blot as shown in Fig. 9. Unbound, excess PrP-res$^{3F4}$ in the supernatant was effectively washed from the cells by four rinses of fresh media (Fig. 9) although some PrP-res$^{3F4}$ associated with detached cells, cell debris, or unbound brain homogenate was observed in the cellular supernatant (Fig. 9, SN pellet). Significant amounts of PrP-res$^{3F4}$ remained cell-associated (Fig. 9, cells). However, unbound PrP-res$^{3F4}$ was still in large excess when compared to cell-associated PrP-res$^{3F4}$ (data not shown) suggesting that the majority of the PrP-res$^{3F4}$ in the brain homogenate did not interact with the cells. No PrP-res$^{3F4}$ could be detected in cells exposed to Mock(3F4) brain homogenate (Fig. 9, lanes 1, 9, and 17). The lack of PrP-res$^{3F4}$ in the rinses following removal of the brain homogenate demonstrates that residual, unbound PrP-res$^{3F4}$ could be removed from the cell monolayer and suggested that the remaining PrP-res$^{3F4}$ was cell-associated.

3.4 Rapid PrP-res$^{3F4}$ uptake into cells is independent of scrapie strain, cell type and host cell PrP-sen expression

In order to determine the kinetics of PrP-res$^{3F4}$ uptake into cells during exposure to TSE infectivity, MoL42-CFD5 cells were exposed to PrP-res$^{3F4}$ in 22L, ME7, or Obihiro scrapie infected brain homogenates for 0-72 hrs. For each strain, cell-associated PrP-res$^{3F4}$ was detected by 2 hours post-exposure and the amount of PrP-res$^{3F4}$ internalized by the cell increased over time up to 72 hours post exposure (Fig. 10A).
**Figure 9: Mouse neural cells take up PrP-res$^{3F4}$**. Western blot analysis of PrP-res$^{3F4}$ uptake into MoL42-CFD5 cells. Equivalent amounts of brain homogenate from 22L scrapie-infected Tg(WT-E1) mice were PK treated and used as positive control (lanes 2, 10, 18) while PK treated brain homogenate from mock-infected Tg(WT-E1) mice was used as negative control (lanes 1, 9, 17). Brain homogenates were added to MoL42-CFD5 cells for 24 hours and then removed from the cell monolayer. Cell debris and dead cells were spun out of the supernatant and the supernatant pellet was PK treated (SN Pellet). Cells were then rinsed with PBS four times (rinse 1-4), lysed, and both the cell lysate and rinses were PK treated. PrP-res$^{3F4}$ was detected in the lysed and PK treated cells (Cells). All blots were analyzed using the mouse monoclonal antibody 3F4 and developed using ECL (Amersham).
Figure 10: Kinetics of PrP-res$^{3F4}$ uptake into cells. A) Representative Western blot analysis of PrP-res$^{3F4}$ uptake into MoL42-CFD5 cells from 0 - 72 hrs. Scrapie-infected brain homogenates from 22L(3F4), ME7(3F4) or Obi(3F4) were analyzed. The first three lanes of each gel show PK-treated scrapie-infected brain homogenate for the appropriate strain loaded in 20 ul, 10 ul, and 5 ul volumes. These lanes were used as internal standards to establish a constant film exposure time from experiment to experiment (4min) in order to quantify the data within the linear range of the film. The amount of PrP-res internalized by the cells increased from 0 to 72 hrs. B) Graphical representation of total cell-associated 22L(3F4), ME7(3F4), or Obi(3F4) taken up by
either MoL42-CFD5 cells (solid lines N=5 or 6) or CF10 cells (dotted lines N=3) from 0-72 hrs. The error bars represent SEM. No statistically significant differences were found between the scrapie strains (\(p>0.05\), Two-way ANOVA) or between the different cell types (\(p>0.05\), Mann Whitney test) for each scrapie strain. C) Graphical representation of total cell-associated 22L, ME7 or Obihiro PrP-res\(^{3F4}\) taken up by MoL42-Ψ2A2 fibroblast cells (N=3) from 0-72 hrs where the error bars represent SEM. No statistically significant differences were found between the scrapie strains (\(p>0.05\), Two-way ANOVA) or between the fibroblast and neuronal cell types (\(p>0.05\), Mann Whitney test). All blots were analyzed using the mouse monoclonal antibody 3F4. For all data, the amount of PrP-res is expressed in pixels and was quantified by analysis of ECL developed western blots using the UN-SCAN-IT software as detailed in the Methods.
Quantification of total PrP-res$^{3F4}$ uptake into the cells for each given time point showed that there were no significant differences in PrP-res$^{3F4}$ uptake amongst the different scrapie strains. For each strain, approximately half of the total PrP-res$^{3F4}$ taken up during the duration of the experiment was taken up between 10-15 hrs. Although ME7 appeared to be taken up more slowly by MoL42-CFD5 cells (Fig. 10B, ME7 solid line), this difference was not statistically significant when compared to the other strains (p>.05 by 2-Way ANOVA).

Previous studies have suggested that cells exposed to scrapie associate with PrP-res independently of host cell PrP expression ($^{34,53}$). However, given that both the Rov ($^{53}$) and CHO ($^{34}$) cell lines used in previous studies may still express low levels of endogenous PrP-sen ($^{34}$, and S. Priola unpublished data), a role for PrP-sen in PrP-res uptake could not be completely ruled out. To determine whether or not host cell PrP-sen expression was needed for the internalization of PrP-res, we utilized a cell line (CF10) derived from Prp$^{0/0}$ mice. CF10 cells are the parent cell line of MoL42-CFD5 cells but do not express PrP-sen. PrP-res$^{3F4}$ was taken up by the CF10 cells similarly to MoL42-CFD5 cells (Fig. 10B, dashed lines). Thus, the uptake of PrP-res derived from different strains of mouse scrapie is independent of PrP-sen expression by the host cell.

We next determined if cell type could influence PrP-res uptake into cells. To test this, non-neuronal MoL42-$\psi$2A2 cells (a fibroblast cell line) were analyzed using the same kinetics assay employed on both the MoL42-CFD5 and CF10 cell lines. Results from this assay showed that, for all strains tested, the MoL42-$\psi$2A2 cells took up PrP-res$^{3F4}$ from scrapie brain homogenate similarly to either MoL42-CFD5 or CF10 cells.
(Fig. 10C). Thus, the kinetics of PrP-res uptake were similar for both neuronal and non-neuronal cell types, at least with respect to fibroblast cells.

3.5 Increased PrP-res uptake correlates with increased cell number

The observed increase in cell-associated PrP-res$^{3F4}$ over 72 hours could be due either to an increase in total cell number or an increase in the amount of PrP-res$^{3F4}$ per cell. To determine if increased PrP-res$^{3F4}$ uptake correlated with cell number, the number of cells at each time point was divided by the amount of PrP-res$^{3F4}$ taken up by the cells as quantified by Western blot. This ratio of cell number to cell-associated PrP-res$^{3F4}$ was plotted against the time cell cultures were exposed to scrapie brain homogenate. For all strains and cell lines tested, the ratio of cells to total PrP-res$^{3F4}$ was high during the first 6 hours of exposure to infected brain homogenate (Fig. 11A-C). However, over time that ratio decreased and, as indicated by the linear portion of the curve, after 8 hours was essentially 1:1. These data suggest that the system had reached a saturation point and that the population of cells within the culture that could take up PrP-res was limited (Fig. 11A-C).
Figure 11: Increased levels of cellular PrP-res$^{3F4}$ correlate with increased cell number. Ratio of total cell number to total cell-associated PrP-res$^{3F4}$ for scrapie strains 22L(3F4), ME7(3F4) or Obi(3F4). PrP-res$^{3F4}$ was quantified using ECL developed western blots analyzed with the UN-SCAN-IT software. A) Ratio of the number of MoL42-CFD5 cells to total PrP-res$^{3F4}$ (N=5). B) Ratio of the number of CF10 cells to total PrP-res$^{3F4}$ (N=6). C) Ratio of the number of MoL42-ψ2A2 cells to total PrP-res$^{3F4}$
(N=6). The variability of the data at later time points was likely due to increased cell death over time (data not shown) rather than any cell-type differences.
3.6 Kinetics of PrP-res$^{3F4}$ uptake into mouse neural cells by immunofluorescence

In order to validate the western blot data from Figure 10 and determine if the number of PrP-res$^{3F4}$ positive cells increased over time, the uptake of partially purified PrP-res$^{3F4}$ into MoL42-CFD5 cells was assessed by immunofluorescent microscopy for the scrapie strains 22L(3F4), ME7(3F4) and Obi(3F4) (Fig. 12). Similar to the western blot data in Fig. 10, cell-associated PrP-res$^{3F4}$ was detected as early as 2 hours. PrP-res$^{3F4}$ was observed as small, punctate aggregates generally localized to both cytoplasmic and perinuclear regions (Fig. 12). Consistent with the data in Figure 11, as cell numbers increased so too did the number of cells positive for PrP-res$^{3F4}$. However, for all time points examined, only 30%-40% of the cells were positive for PrP-res$^{3F4}$ (Fig. 13). Our results suggest that there is a select subpopulation of cells able to take up PrP-res which, over time, divide and increase in number leading to the observed increase in the amount of cell-associated PrP-res.
Figure 12: PrP-res\textsuperscript{3F4} uptake into neural cells using immunofluorescence microscopy. Partially purified PrP-res\textsuperscript{3F4} from scrapie strains 22L(3F4), ME7(3F4), Obi(3F4) or mock controls was added to MoL42-CFD5 cells for 2 – 48 hrs. Cells were rinsed, fixed and immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse FITC labeled antibody (green) was used to detect PrP-res\textsuperscript{3F4} while DAPI stain (blue) denotes cell nuclei. All images were taken with a 40X objective.
22L(3F4)

2 hrs  12 hrs  48 hrs
ME7(3F4)

2 hrs

12 hrs

48 hrs
Obi (3F4)

2 hrs     12 hrs

48 hrs
**Figure 13: Percentage of cells positive for PrP-res\textsuperscript{3F4} from 0 - 48 hours post exposure.** Graphical representation of the percentage of cells with detectable internalized PrP-res\textsuperscript{3F4} from 0 - 48 hours post exposure from scrapie strains 22L(3F4), ME7(3F4) or Obihiro(3F4). No statistically significant differences were found between the scrapie strains ($p>0.05$, *Two-way ANOVA*)
3.7 PrP-res is internalized by cells

To determine if cell-associated PrP-res$^{3F4}$ was bound to the cells non-specifically or internalized via an active cellular process such as endocytosis, MoL42-CFD5 cells were exposed to scrapie-infected brain homogenates and incubated either at 18°C, which blocks endocytosis, or at 37°C. As shown in Fig. 14, cells exposed to scrapie-infected brain homogenates at 37°C were positive for PrP-res$^{3F4}$ while cells which were exposed to scrapie-infected brain homogenates at 18°C were negative for PrP-res$^{3F4}$. Thus, within the sensitivity of the assay, our data suggest that cell-associated PrP-res$^{3F4}$ is likely being actively internalized by the cells.

To further validate that PrP-res$^{3F4}$ is internalized and not merely adhering to the cell surface, a series of compiled z-stack images were collected by confocal microscopy on MoL42-CFD5 cells that had been exposed to partially purified PrP-res$^{3F4}$ for 24hrs (Fig. 15). Whether the cell images are rotated about the Y-axis (Fig. 15A) or visualized by select Z-plane (Fig. 15B), PrP-res$^{3F4}$ is clearly detected between the upper and lower planes of the cell membrane. The data support the conclusion that PrP-res$^{3F4}$ is being internalized by the cells.

3.8 Co-localization of PrP-res with select endocytosis markers

To more thoroughly assess where inoculum PrP-res could be located within the cell, co-localization of PrP-res$^{3F4}$ with a variety of endocytosis markers was examined by confocal microscopy. Cells were exposed for 2, 12, or 24 hrs to PrP-res$^{3F4}$ and stained for PrP-res$^{3F4}$ and a panel of different endocytosis markers. As shown in Fig. 16 PrP-res$^{3F4}$ co localization with select endocytosis markers was determined by examining all
Z-plane images for color overlap. In all images, PrP-res\textsuperscript{3F4} was labeled with Alexa-Fluor-488 (green) and the endocytosis marker of choice was always labeled with Alexa-Fluor-596 (red) providing orange color for all points of co localization. Cholera toxin (subunit B) is a general cell surface marker that also accumulates in the Golgi apparatus. Only about 5% of this marker associated with the PrP-res\textsuperscript{3F4} at any time point. Co-localization was only occasionally found at the cell surface, suggesting that, with respect to the first 24 hrs, PrP-res\textsuperscript{3F4} does not accumulate in the Golgi apparatus (Fig. 16). Calnexin is a general endoplasmic reticulum (ER) marker, and no co-localization was observed with it and PrP-res\textsuperscript{3F4} for any time point (Fig. 16), suggesting that inoculum PrP-res\textsuperscript{3F4} is not trafficked to the ER. A marker for early endosomal antigen 1 (EEA1) was used to identify premature lysosomes and endosomes. PrP-res\textsuperscript{3F4} was not found to localize with EEA1 (Fig 16). LAMP1 and LAMP2 are late stage endosomal or lysosomal markers. Approximately 5-10% of PrP-res\textsuperscript{3F4} was found to associate with either LAMP1 or LAMP2 suggesting that some PrP-res\textsuperscript{3F4} was trafficked through lysosomes. This is consistent with other studies showing that, in persistently infected cells, PrP-res accumulates in endolysosomes (24, 23).

The final maker assessed for co-localization with PrP-res\textsuperscript{3F4} was γ-tubulin. γ-tubulin is a major component of the microtubule organizing center (MTOC). The MTOC or centriole is a major anchoring point by which microtubule extensions elongate. Both Golgi and ER are concentrated at the MTOC as it provides structural scaffolding for them (66, 47). Many viruses such as the Mason-Pfizer monkey
Figure 14: PrP-res$^{3F4}$ uptake into neural cells is blocked at 18°C. MoL42-CFD5 cells were exposed to infected brain homogenate from 22L(3F4), ME7(3F4) or Obi(3F4) scrapie for 24 hours at either 37°C or 18°C. Non-PK digested infected brain homogenates were run to illustrate total PrP levels (lanes: 1, 7, 13) while PK-digested brain homogenates were run as a positive control for PrP-res (lanes: 2, 8, 14). PrP was taken up by cells at 37°C (-PK lanes 3, 9, 15). PrP-res$^{3F4}$ was detected in cells which had been exposed to scrapie brain homogenate and incubated at 37°C (+PK lanes 4, 10, 16). However, little or no PrP (-PK lanes 5, 11, 17) and no PrP-res$^{3F4}$ (+PK lanes 6, 12, 18) was detected in cells which had been exposed to scrapie brain homogenate and incubated at 18°C. Samples in lanes 2-6, 8-12 and 14-18 were PNGaseF treated to remove all PrP-res glycosylation. All blots were analyzed using the mouse monoclonal antibody 3F4 and developed using ECL (Amersham).
Figure 15: Confocal Z-stack series of acutely infected MoL42-CFD5 cells. Fixed MoL42-CFD5 cells were exposed to partially purified 22L(3F4) PrP-res$^{3F4}$ for 24 hours and scanned by confocal microscopy to generate a Z-stack series. A) Compiled Z-stack series rotated about the Y-axis or B) Select Z-planes. All cells were immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse Alexa-Flour-488 labeled antibody (green) was used to detect PrP-res$^{3F4}$ while Alexa-Flor-596 conjugated cholera toxin subunit B (red) labels the cell surface and DRAQ-5 stain (blue) denotes cell nuclei. All images were taken with a 63X objective and 2X dynamic zoom.
Figure 16:
Fixed CF10 cells were exposed to partially purified 22L(3F4) PrP-res$_{3F4}$ for either 2, 12 or 24 hours, labeled for select endocytosis markers and scanned by confocal microscopy. Cells treated with an uninfected PrP$_{3F4}$ partially purified brain preparation was used as a PrP-sen control (Mock). All cells were immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse Alexa-Flour-488 labeled antibody (green) was used to detect PrP-res$_{3F4}$ while Alexa-Fluor-596 was used to label either cholera toxin (subunit B), calnexin, EEA1, LAMP1, LAMP2 or MTOC (red). DRAQ-5 stain (blue) denotes cell nuclei. The confocal plane that best displayed the select endocytosis marker was chosen for the above images. All images were captured with a 40X objective and 2X dynamical zoom.
Mock

2 hrs     12 hrs

24 hrs
Cholera Toxin (Subunit B)

2 hrs     12 hrs

24 hrs
Calnexin

2 hrs     12 hrs

24 hrs
EEA1

2 hrs     12 hrs

24 hrs
LAMP1

2 hrs  

12 hrs

24 hrs
LAMP2

2 hrs     12 hrs

24 hrs
MTOC

2 hrs  12 hrs

24 hrs
virus assemble at the MTOC \cite{69} and it was possible that PrP-res\textsuperscript{3F4} was being trafficked there. However, PrP-res\textsuperscript{3F4} did not appear to localize with MTOCs (Fig. 16). Findings from these experiments are summarized in Table 4.

3.9 PrP-res aggregate size influences PrP-res binding and uptake

The rate of uptake of PrP-res\textsuperscript{3F4} in cells in our assay was quite different from that of previous studies where large, fluorescent PrP-res aggregates were not fully internalized by SN56 neuronal cells until several days post PrP-res exposure \cite{3, 43}. To test whether differences in cell type or PrP-res preparation could account for these discrepancies, we exposed differentiated SN56 neuronal cells for 24 hrs to either 22L PrP-res\textsuperscript{3F4} or 22L PrP-res\textsuperscript{3F4} conjugated to an Alexa-Fluor-596 fluorescent tag (Fig. 17). SN56 cells exposed to Alexa-Fluor labeled 22L PrP-res\textsuperscript{3F4} showed very large PrP-res\textsuperscript{3F4} aggregates (Fig. 17A) that were indistinguishable from those described previously \cite{3, 43}. By contrast, SN56 cells exposed to non-Alexa-Fluor labeled 22L PrP-res\textsuperscript{3F4} (Fig. 17B) showed the same small, punctate aggregates of PrP-res\textsuperscript{3F4} observed in MoL42-CFD5 cells (Fig. 12). When both Alexa-Fluor labeled PrP-res from 22L scrapie-infected wild type mice and non-Alexa-Fluor labeled 22L PrP-res\textsuperscript{3F4} were added to the same monolayer of SN56 cells, the difference in PrP-res aggregate size was even more apparent (Fig. 17C). The difference in aggregate size between 22L PrP-res and Alexa-Fluor labeled 22L PrP-res strongly
<table>
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<th>Marker</th>
<th>Organelle Labeled</th>
<th>% Co-localization</th>
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<tr>
<td>Cholera Toxin (Subunit B)</td>
<td>Cells Surface / Golgi</td>
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<tr>
<td>Calnexin</td>
<td>Endoplasmic Reticulum</td>
<td>0%</td>
</tr>
<tr>
<td>Early Endosomal Antigen 1</td>
<td>Endosomes</td>
<td>0%</td>
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<tr>
<td>LAMP1</td>
<td>Lysosomes</td>
<td>≈ 5 – 10%</td>
</tr>
<tr>
<td>LAMP2</td>
<td>Lysosomes</td>
<td>≈ 5 – 10%</td>
</tr>
<tr>
<td>MTOC</td>
<td>Centriols</td>
<td>0%</td>
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</tbody>
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Table 4: Co-localization of different cellular organelle markers with host cell associated inoculum PrP-res\(^{3F4}\).
Figure 17: Large PrP-res aggregates bind to the cell surface and are not rapidly internalized. Differentiated SN56 cells were exposed to Alexa-Fluor-596 labeled (red) or unlabeled (green) 22L PrP-res$^{3F4}$ for 24 hours. Cells were then rinsed, fixed and when necessary, immunolabeled with the mouse monoclonal antibody 3F4 (Panels B-D). Anti-mouse FITC labeled antibody (green) was used to detect 22L PrP-res$^{3F4}$, while DAPI stain (blue) denotes cell nuclei. Alexa-Fluor-596 labeled proteins are red. All images were taken with a 40X objective. Cells were exposed to A) Alexa-Fluor labeled partially purified 22L PrP-res$^{3F4}$ (red), B) partially purified 22L PrP-res$^{3F4}$ (green), C) Alexa-Fluor labeled partially purified PrP-res from a 22L scrapie-infected wild type mouse (red) and partially purified 22L PrP-res$^{3F4}$ (green) (inset taken with a 60X objective). In panel D, cells were exposed for 24 hours at either 18°C or 37°C to Alexa-Fluor labeled partially purified PrP-res from a 22L scrapie-infected wild type mouse (red) and 22L PrP-res$^{3F4}$ (green). The larger Alexa-Fluor labeled PrP-res aggregates do not co-localize with the smaller, more punctate PrP-res$^{3F4}$ aggregates.
suggests that the larger PrP-res aggregates were primarily a consequence of the Alexa-Fluor labeling process.

When both Alexa-Fluor labeled 22L PrP-res from scrapie-infected wild type mice and non-Alexa-Fluor labeled 22L PrP-resSuperscript 3F4 were added to the same monolayer of SN56 cells there was almost no co-localization between the PrP-res molecules in the two preparations (Fig. 17C). To help resolve this difference in co-localization, we conducted a temperature shift analysis of SN56 cells exposed to both Alexa-Fluor labeled 22L PrP-res from wild type mice and non-Alexa-Fluor labeled PrP-resSuperscript 3F4 (Fig. 17D). Cells incubated at 37°C (Fig. 17D) showed localization patterns similar to Figure 17C. However, consistent with the Western blot data in Figure 9, cells incubated at 18°C were negative for PrP-resSuperscript 3F4 once again suggesting that PrP-resSuperscript 3F4 was not internalized by the cells (Fig. 17D). By contrast, Alexa-Fluor labeled PrP-res was cell-associated at 18°C (Fig. 17D) suggesting that it was bound to the cell surface. Our data suggest that during the first 24 hrs of infection, large PrP-res aggregates are localized primarily at the cell surface while small aggregates are rapidly internalized.

3.10 Infectious brain homogenate PrP-res is taken up most efficiently by cells

A number of PrP-res preparation methods have been employed to infect cells in vitro (3, 4, 5, 78). To test if different PrP-res preparation methods would alter PrP-res uptake into cells, equal amounts of scrapie strain Obihiro PrP-resSuperscript 3F4 derived either from partially purified PrP-res or infectious crude brain homogenate was added to MoL42-CFD5 cells and the uptake of PrP-res into the cells was assayed by Western blot. By 8 hours post infection, infectious crude brain homogenate appeared to be taken up
significantly more efficiently then partially purified PrP-res (Fig. 18A, open triangles).

To determine whether or not this discrepancy in PrP-res uptake was due to a difference in total protein, mock infected brain homogenate was added to the partially purified PrP-res in order to match the total protein content found in the infectious brain homogenate. Protein adjusted partially purified PrP-res was taken up with the same efficiency as partially purified PrP-res alone (Fig. 18A). Microsome PrP-res was also taken up by cells with the same efficiency as either partially purified PrP-res or total protein adjusted partially purified PrP-res (total protein adjusted with mock infected microsome preparation) (Fig. 18B). Taken together, our results suggest that there is an increased efficiency in the uptake of PrP-res when it is associated with an infectious brain homogenate.
Figure 18: Infectious brain homogenate PrP-res$^{3F4}$ is taken up more efficiently than either microsome or partially purified PrP-res$^{3F4}$. Graphical representation of PrP-res$^{3F4}$ uptake from 0 - 24 hrs into MoL42-CFD5 cells (N = 6) where error bars represent SEM. A) Kinetics of PrP-res$^{3F4}$ uptake using either infectious brain homogenate PrP-res$^{3F4}$ (BH-I), partially purified PrP-res$^{3F4}$ (PrP-res$^{3F4}$) or partially purified PrP-res$^{3F4}$ with mock infected brain homogenate (PrP-res$^{3F4}$ + BH-M) added to match the total protein content of BH-I (* p<0.05, t = 8-24 hrs, Bonferroni test). B) Kinetics of PrP-res$^{3F4}$ uptake using either infectious microsome PrP-res$^{3F4}$ (Microsome-I), purified PrP-res$^{3F4}$ (PrP-res$^{3F4}$) or partially purified PrP-res$^{3F4}$ with mock infected microsomes (PrP-res$^{3F4}$ + Microsome-M) added to match total protein content of infectious microsomes (p>0.05 for all time points, Bonferroni test). All data was obtained with IR-dye 800CW developed western blots where the PrP-res$^{3F4}$ level was quantified as fluorescent units using the Li-Cor Odyssey imaging system and associated software.
Chapter 4

DISCUSSION

4.1 Acute uptake of PrP-res

The acute development of TSE infection in host tissue occurs through TSE agent/host interactions which remain poorly understood. The consequence of these interactions may help determine disease susceptibility, pre-clinical incubation time, and later stage pathology. Previous in vitro studies have shown that many cell types initially propagate PrP-res although only certain combinations of cell types and strains can maintain infection indefinitely (79). While this early work studied the conversion of host PrP-sen to PrP-res and established select boundaries for infectivity, the fate of the PrP-res inoculum used to infect the cells remained unclear (79). It is important to understand how PrP-res from an exogenous source interacts with the cell during the early stages of infection given that different strains of inoculum PrP-res might interact with different host cells differently. These differences may determine how long term persistent infection is maintained following the acute stage of TSE infection. Furthermore, such data might provide insight into how host cells process and interact with inoculum PrP-res and thus help to explain cell susceptibility to infection.

The purpose of this study was to develop and employ a method for uniquely detecting inoculum PrP-res in order to help assess early interactions of the TSE agent with the host cell. PrP-res tagged with a unique antibody epitope has allowed us to examine for the first time the cellular uptake of infectious inoculum PrP-res in vitro in the absence of background from host cell derived PrP-res or PrP-sen. We have been able to
follow the fate of the inoculum by both Western blot (Fig. 9) and immunofluorescence microscopy (Fig. 5, 12). Our data show that acute PrP-res uptake (i.e. within the first 72 hrs) is both cell type and scrapie strain independent and are consistent with previous work where the acute conversion of cellular PrP-sen to PrP-res was also found to be cell type and scrapie strain independent (79). Furthermore, this study has demonstrated that during the first three days post-scrapie exposure cells take up PrP-res from different strains with similar kinetics (Fig. 10, Fig. 12) and that this process does not require host cell expression of PrP-sen (Fig. 10). Thus, although PrP-sen is necessary for persistent PrP-res formation and scrapie infection, its absence in cells does not inhibit the cells ability to take up PrP-res. Overall, our results suggest that most events early during mouse scrapie infection (i.e. the first 72 hrs) will likely be similar for all strains and cell types in vitro.

Whether evaluated by Western blot (Fig. 9) or by confocal microscopy (Fig. 15) PrP-res is internalized by mouse cells. This internalization or uptake is mediated by an active cellular process. When cells were exposed to PrP-res and incubated at 18°C (Fig. 14, Fig. 17D) no PrP-res was taken up by the cells. This suggests that mouse PrP-res is taken up into cells through an active cellular process which may not necessarily be mediated by a specific cell ligand. Alternatively, it is possible that the necessary receptor for internalization of mouse PrP-res is absent from the cell surface when the cells are incubated at 18°C. However, previous studies using the hamster scrapie strain 263K and Chinese hamster ovary cells found that hamster PrP-res remained cell associated at 18°C and likely bound to cells via cell surface heparan sulfate (34). Thus, it was somewhat surprising that we found no evidence that mouse PrP-res\textsuperscript{3F4} was cell-associated at 18°C (Fig. 14, Fig. 17D). Although, consistent with the results reported by Hajazi et al.,
preliminary studies in our laboratory also suggest that at least some hamster 263K PrP-
res can associate with cells at 18ºC (data not shown).

Regardless of strain, PrP-res uptake into cells was detectable by 2 hrs (Fig. 10, 11, 12) and was more rapid for the first 8 hours post-infection when compared to later time points (Fig. 11). After 8 hrs, PrP-res uptake into cells was apparently restricted by total cell number. This change in the kinetics curve may be related to the fact that most cells are still rapidly dividing and in log phase during the first 8 hours of exposure to PrP-res. Over time, the cells become more confluent and PrP-res uptake may be reduced as cellular division rates slow. This interpretation is consistent with the recent observation that cell division can also influence PrP-res levels within mouse neuroblastoma cells persistently infected with scrapie (31).

Interestingly, at any given timepoint only 30-40% of the cells were able to take up detectable levels of PrP-res (Fig. 13) despite the fact that PrP-res was always supplied in excess. One possible explanation for this result is that there is a limited population of susceptible cells which can detectably take up PrP-res. Over time, these cells divide and increase in number leading to the observed increase in the amount of cell-associated PrP-res, a process that may also be important once persistent infection has been established (31, 81). Additionally, it may be that only certain PrP-res molecules can be efficiently taken up by the cell. The recent observation that PrP-res particle size can influence scrapie infectivity (71), the demonstration that large Alexa-Fluor PrP-res aggregates are broken down over several days into smaller aggregates which are then internalized by the cell (43), as well as our data demonstrating that large AlexFluor-labeled aggregates are not rapidly internalized (Fig. 17A), are all consistent with this interpretation. Thus, PrP-res
aggregate size likely influences both the initial interaction of PrP-res with the cells as well as PrP-res uptake.

Mouse PrP-res from all strains tested formed small, punctate aggregates that appeared to localize primarily in the perinuclear and cytoplasmic regions of acutely infected cells (Fig. 6, 8, 11). This localization is consistent with earlier studies of PrP-res in persistently infected cells (24, 48, 54, 75) and these punctate aggregates likely act as PrP-res seeds that help propagate new PrP-res formation (79). Upon further examination, PrP-res was associated with endocytosis markers LAMP1 and LAMP2 (5-10%) (Fig. 16). However, almost no PrP-res associated with the Golgi apparatus, endoplasmic reticulum, microtubule organizing center or early endosomes within the first 24 hrs of infection (Fig. 16). This might suggest that PrP-res is taken up through general mechanisms of pinocytosis. It should also be noted that host generated PrP-res evaluated in the chronically infected L929-3F4 cell line (Fig. 5) was distributed differently from the infected inoculum PrP-res. In future studies it would be of value to test these persistently infected cells to see if endogenously produced PrP-res is localized differently from exogenously added PrP-res.

Despite the evidence that cell-type can influence the establishment of a persistent infection (79), the general localization of PrP-res was the same for all cell types and strains tested. For example, both ME7 and 22L PrP-res appeared to be in similar cytoplasmic and perinculear locations (Fig. 12), despite the fact that the 22L scrapie strain infects fibroblast cells while ME7 does not (79). This suggests that the initial cellular location of the inoculum PrP-res may not be a determining factor in whether or not a cell becomes persistently infected.
Assessing inoculum PrP-res for physical differences, it is now clear that with respect to predominant density and resistance to PK digestion scrapie strains 22L(3F4), ME7(3F4) and Obihiro(3F4) are quite similar (Fig 6). This suggests that although different PrP-res strains may contain differing conformations ($^{28,67, 64}$), they can not always be independently assessed via PK sensitivity or by predominant density. These physical similarities in strain may in part explain why no differences were observed in their respective ability to acutely infect cells (Fig. 10, 12).

Although different strains of PrP-res appear to exhibit similar physical characteristics, how they are prepared can affect cellular uptake. PrP-res within an infectious brain homogenate was taken up more efficiently then either partially purified PrP-res or PrP-res in microsome preparations (Fig. 18). This suggests that there may be a co-factor or some type of PrP-res associated microenvironment that is specific to infectious brain homogenate which is removed or disrupted during either of the alternative PrP-res preparations. Consistent with the idea that PrP-res may have to be in a specific microenvironment to efficiently infect cells ($^3$), the simple addition of brain homogenate protein to partially purified PrP-res preparations did not enhance the uptake of partially purified PrP-res (Fig. 18A). It is also unlikely that microsomes provide the appropriate microenvironment given that microsome PrP-res was not taken up as efficiently as PrP-res in brain homogenate (Fig. 18B). Our results do not negate the possibility that microsomes may be more infectious ($^3$), but suggest that the apparent increased efficiency with which microsomes infect cells is not due to more efficient uptake of PrP-res during the early stages of infection as previously proposed ($^3$).
4.2 Implications and future directions

In combination with earlier studies (79), our data suggest that there may be at least two blocks to the persistent infection of a cell with scrapie. The first occurs during the acute stage of infection (0-72hrs) when factors such as PrP-res aggregate size, the microenvironment of the PrP-res inoculum, and/or the presence of a specific population of cells help to determine whether or not PrP-res is bound to the cell and how it is internalized. This block would not be strictly dependent upon scrapie strain or cell type but rather would depend upon heterogeneity in both the PrP-res and cell populations. Once a cell culture becomes persistently infected, cellular heterogeneity may also influence both the level and stability of infectivity over long term passage (15, 30, 81).

Previous work has shown that the mouse Ψ2 fibroblast cells used in the present study can be infected with the 22L, but not the ME7, strain of mouse scrapie (79). The fact that ME7 acts similarly to 22L during acute infection (Figs. 10, 12 79) suggests that the second block to infection is scrapie-strain dependent and occurs 1) after uptake and localization of the inoculum PrP-res and, 2) after an acute burst of new PrP-res formation in the cell (79).

Looking beyond the scope of this study, it is easy to see where the ability to uniquely detect inoculum PrP-res might be useful in vivo. With this tool it should be possible to inject PrP-res into a host organism, and subsequently track where the material traffics and potentially binds within the host. Studies are currently underway to determine if inoculum PrP-res injected either intrathecally (i.e. into the spinal cord) or into the sciatic nerve can be tracked in a mouse over a 72 hr period. This information would provide useful insight into the acute stage of TSE infection in vivo. Conversely, if
it is possible to track the PrP-res$^{3F_4}$, then it should be possible to inject wild-type PrP-res into mice over expressing mouse PrP$^{3F_4}$ (Tg(WT-E1) mice). This should enable us to determine the sites of new PrP-res formation, which will advance our understanding of the mechanisms underlying TSE disease pathology.
Chapter 5

CONCLUSION

In conclusion, PrP-res internalization by cells is a rapid cellular process which is dependent upon the predominant aggregation state of inoculum PrP-res, the interactive host/inoculum microenvironment, and a subpopulation of susceptible cells. Internalization and trafficking of PrP-res appeared to be independent of cell type, scrapie strain or host expression of PrP. Acute uptake of PrP-res is an active cellular process through yet undefined host mechanisms. Our ability to uniquely detect inoculum PrP-res independent of host PrP should provide greater insight into host/agent interactions that may be critical in establishing host infection. The development of these tools may not only provide useful in vitro data, but may also allow us to understand how PrP-res is trafficked in vivo and identify sites of new PrP-res formation in vivo.


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