1618 *Biochemistry* **2011,** *50,* 1618–1623
DOI: 10.1021/bi1017683

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Histidines in the Octapeptide Repeat of PrP^C React with PrP^{Sc} at an Acidic pH[†]

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Received November 3, 2010; Revised Manuscript Received January 25, 2011

ABSTRACT: Cellular PrP is actively cycled between the cell surface and the endosomal pathway. The exact site and mechanism of conversion from PrP^C to PrP^{Sc} remain unknown. We have previously used recombinant antibodies containing grafts of PrP sequence to identify three regions of PrP^C (aa23−27, 98−110, and 136−158) that react with PrP^{Sc} at neutral pH. To determine if any regions of PrP^C react with PrP^{Sc} at an acidic pH similar to that of an endosomal compartment, we tested our panel of grafted antibodies for the ability to precipitate PrP^{Sc} in a range of pH conditions. At pH near or lower than 6, PrP-grafted antibodies representing the octapeptide repeat react strongly with PrP^{Sc} but not PrP^C. Modified grafts in which the histidines of the octarepeat were replaced with alanines did not react with PrP^{Sc}. PrP^{Sc} precipitated by the octapeptide at pH 5.7 was able to seed conversion of normal PrP to PrP^{Sc} in vitro. However, modified PrP containing histidine to alanine substitutions within the octapeptide repeats was still converted to PrP^{Sc} in N2a cells. These results suggest that once PrP has entered the endosomal pathway, the acidic environment facilitates the binding of PrP^{Sc} to the octarepeat of PrP^C by the change in charge of the histidines within the octarepeat.

Prion diseases, or transmissible spongiform encephalopathies, such as Creutzfeldt−Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and chronic wasting disease (CWD) in cervids, are a group of fatal neurodegenerative diseases caused by the conversion of the normal cellular prion protein, PrP^C,[1] to the misfolded disease associated form, PrP^{Sc} (*1*−*5*). The exact mechanisms of replication and pathogenesis of these spontaneous, heritable, and infectious diseases still remain poorly understood (*6*). Evidence has been shown that the direct and specific interaction of PrP^C with PrP^{Sc} is vital for the formation of nascent PrP^{Sc} from endogenous PrP^C (*7*, *8*). Interestingly, PrP^C and PrP^{Sc} have the same amino acid sequence and differ only in secondary structure (*9*−*12*). While cellular PrP^C is composed primarily of α-helical structure, is soluble in mild detergent, and is sensitive to digestion with proteinase K, PrP^{Sc} is rich in *β*-sheet structure, is insoluble in nondenaturing conditions, and is partially resistant to proteinase K digestion (*10*, *13*−*15*).

Mature full-length PrP is composed of amino acids 23−231. The C-terminal half of PrP^C contains three α-helices and two short *β*-strands. The N-terminal region however is largely unstructured and flexible (*16*). While the protease-resistant fragment of PrP^{Sc}, composed of residues 90−231, retains the ability to transfer disease, the N-terminal domain has been shown to modulate disease progression in vivo and influence higher order aggregation in vitro (*2*, *17*, *20*, *21*).

The N-terminal domain of PrP contains a stretch of highly conserved octapeptide repeats, in which the amino acid sequence P(Q/H)GGG(G/−)WGQ is repeated in tandem five times, four of which are PHGGGWGQ. Amplifications of the octarepeat from the normal 5 to as many as 14 are associated with heritable CJD, Gerstmann−Straussler−Scheinker disease (GSS), and fatal familial insomnia (FFI) (*18*). The rate of formation of protease-resistant PrP has also been shown to increase as the result of additional copies of octarepeat (*19*). Mice lacking all five copies of octarepeats show longer incubation times compared to wild-type mice when infected with scrapie and do not display the histopathology typical of TSE (*20*). However, the retention of a single copy of the octapeptide is sufficient to sustain the replication of the disease-associated isomer and development of disease (*20*). Furthermore, the involvement of the octarepeats has been shown to stabilize PrP^C−PrP^{Sc} interactions in vitro (*33*).

PrP is exported to the cell surface as a non-, mono-, or diglycosylated glycosylphosphatidylinositol-anchored protein. The protein is then cycled between the cell surface and the endocytic pathway (*22*−*26*). The N-terminus of PrP has been shown to be essential for the uptake of PrP into endosomes via clathrin-coated pits, possibly by the binding of a receptor (*26*).

Flanking the octarepeats of PrP are two charged regions, CC^1 and CC^2 (aa23−27 and aa95−110), each containing four positively charged residues (*6*). We have previously shown that these charged regions of PrP^C specifically react with PrP^{Sc} at pH 7.4 (*27*). In this work we show that the octarepeat region, positively charged at a pH less than 6, also reacts specifically with PrP^{Sc}.

MATERIALS AND METHODS

Preparation of Motif-Grafted Antibodies. Motif-grafted antibodies were generated as previously described (*27*, *28*). Briefly, with the use of overlap extension PCR, sequential

[†]This work was supported, in whole or in part, by National Institutes of Health Grants AG004243, NS053504, and NS046037 and Department of Defense Grant DAMD 17-03-1-0511.

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[1]Abbreviations: PrP, prion protein; PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; OPR, octapeptide repeat; PMCA, protein misfolding cyclic amplification.

 Published on Web 01/26/2011

overlapping 24-mer polypeptides of mouse PrP sequence, collectively representing amino acid residues 19−231, were each grafted into the antibody b12 protein scaffold, replacing native heavy chain complementary-determining region 3 (HCDR3) of the original antibody. Using this approach, a panel totaling of 20 different PrP-grafted antibodies was generated. An additional PrP antibody (OPR) was also prepared, composed of PrP residues PHGGGWGQ, representing a single PrP octapeptide (OPR). Antibodies containing the 59−82 and octapeptide scrambled amino acidic sequence were also generated, as well as antibodies in which the histidine residues present in the segment 59−82 and in the octapeptide were changed to alanine residues.

Antibody Expression and Purification. Antibody expression and purification were performed as previously described (*27*). Antibody expression was carried out by transient transfection of a suspension of 293 human embryonic kidney cells in a serum-free medium (Freestyle 293 expression system; Invitrogen, Carlsbad, CA) with the pDR12 vector containing the b12 light chain gene and PrP-grafted b12 human γ1 heavy chain sequences. Generally, 30 μg of plasmid DNA suspended in Freestyle 293 expression medium was used to transfect a total of 3×10^7 293 cells at a final cell density of 1×10^6 cells/mL. If necessary, to enhance antibody expression, 293 cells were cotransfected with 5 μg of pAdVantage vector (Promega, Madison, WI). Transfected cells were incubated at 37 °C in a humidified atmosphere of 8% CO_2 with rotation at 125 rpm. Cells were harvested 48−72 h posttransfection, and the culture supernatant was cleared by centrifugation. Antibody was purified from clarified supernatant over a clone-dedicated protein G Sepharose column, eluted from the resin in the presence of 0.1 M citric acid, pH 3.0, brought to neutral pH with addition of 2 M Tris base (adjusted to pH 8.0 with 37% (w/v) HCl), dialyzed against phosphate-buffered saline, pH 7.4, and then passed through a 0.22 μm filter and stored at −80 °C until needed.

Immunoprecipitation of PrP. Motif-grafted IgG antibodies were used to immunoprecipitate PrP from the brains of RML-prion infected and uninfected mice as previously described (*27*, *28*). To facilitate comparison between different experiments, homogenates prepared from the brains of five different prion infected mice were pooled, aliquoted, frozen at −80 °C, and used throughout the study. Final concentrations of antibody in the immunoprecipitation experiments are noted and varied between 3 and 10 μg/mL. Throughout this study, to reduce the possibility of nonspecific binding interactions between the PrP-grafted antibodies and PrP, binding studies were performed in the presence of a final concentration of 1% (w/v) Triton X-100.

Generation of Octarepeat Histidine to Alanine Mutant PrP. The 3F4 epitope-tagged mutant mouse PrP coding sequence, in which the histidines within the octarepeat region were changed to alanines, was generated by overlap extension PCR and cloned into pCB6+ as previously described (*29*). The template for the PCR reactions was pCB6+ containing wild-type mouse PrP with the 3F4 epitope.

ScN2a Cell Line Transfection and Western Blot. ScN2a cells were transiently transfected with pCB6+ containing epitope-tagged mutant PrP coding sequence as previously described (*29*). In brief, RML infected N2a cells were transfected using Lipofectamine 2000 (Invitrogen). Cells were harvested after 48 h and lysed, and lysates were cleared by centrifugation at 1000*g* before the total protein concentration was determined by BCA (Pierce). Seven hundred micrograms of total protein from each transfection was treated with proteinase K. The PK-treated lysate and 100 μg of untreated lysate were precipitated with methanol−chloroform and run on 12% Tris−glycine gels before being transferred to PVDF membranes. Western blots were performed as previously described. Total PrP was detected by probing with antibody D18. Transfected PrP was detected by probing with antibody 3F4.

Protein Misfolding Cyclic Amplification. Motif-grafted antibodies 89−112, OPR, OPR H-A, and antibody b12 were each directly conjugated to tosyl-activated paramagnetic beads as suggested by the manufacturer (Invitrogen). Twenty-five microliters of each bead preparation was incubated, rotating overnight at 4 °C in 475 μL of PBS and 1% Triton X-100, pH 7.4 or 5.7, plus 25 μL of proteinase K digested 10% RML infected mouse brain homogenate. The beads were then washed 10 times with 1 mL of PBS and 1% Triton X-100, pH 7.4 or 5.7. PrP^{Sc} was eluted from the beads with 25 μL of PBS and 1% Triton X-100, pH 9.

The protein misfolding cyclic amplification procedure was performed as described by Castilla et al. (*34*). PMCA substrate was prepared by making a 10% homogenate of brains from normal PBS and 5 mM EDTA perfused C57BL/6 mice in PBS and 1% Triton X-100 plus protease inhibitors, which was then pooled, aliquoted, and stored at −80 °C. PrP^{Sc} eluted from the antibody-coated beads was diluted 10^{-4} in 200 μL of PMCA substrate. Half of each PMCA sample was subjected to 96 rounds of sonication at 75% power for 20 s and 29 min 40 s of rest at 37 °C using a Misonix S4000 automated sonicator. The remaining half of each sample was stored at −80 °C.

Ten microliters of each sample was digested with proteinase K at 150 μg/mL in PBS, 2% Triton X-100, 2% NP-40, and 2% Tween-20 for 1 h at 37 °C. An equal volume of 2× SDS−PAGE buffer was added, and each sample was heated to 95 °C for 5 min. The samples were then run on a 16% Tris−glycine gel and transferred to a PVDF membrane. Antibody D13 was used to detect PrP.

Nomenclature. Numbering of amino acid residues corresponds to that of Syrian hamster PrP throughout.

RESULTS

An Acidic pH Environment Enhances Selected PrP^C−PrP^{Sc} Interactions. Studies of the subcellular trafficking of PrP^C indicate the protein is rapidly turned over at the cell surface, being internalized via clathrin-coated pits into endocytic structures (*25*, *26*, *30*). The exact site of PrP^{Sc} formation has not yet been unequivocally determined, although PrP^{Sc} has been shown to accumulate in endocytic compartments of the prion-infected cell (*24*, *31*, *32*). As internalized PrP molecules progress through the endocytic pathway, they are exposed to an increasingly acidic pH. The pH of early endosomes is typically near 6, late endosomes near 5, and lysosomes lower still. Thus, the more acidic endosomal environment may potentially exert an important role in prion disease.

Previously, using a large panel of recombinant antibody molecules displaying overlapping peptide grafts collectively spanning PrP residues 19−231, we identified three regions of PrP^C (composed of amino acid residues 23−33, 98−110, and 136−158) that specifically and robustly interact with PrP^{Sc} at neutral pH (*27*). We hypothesize that these PrP sequences likely represent the peptidic components of one flank of the prion replicative interface. To determine if a reduction in pH, to a level similar to that found within early endosomes, could impact

PrP^{C}–PrP^{Sc} interactions, we measured the binding of selected PrP-grafted antibodies to PrP^{Sc} at pH 5.7. As illustrated in Figure 1, at lower pH, binding of IgG 19–33, 89–112, and 136–158 to PrP^{Sc} remained robust and was in some cases even enhanced over that typically seen at pH 7.4 (Figure 1), whereas no PrP binding was detected when using the IgG b12 scaffold at pH 5.7. Similarly, antibodies containing PrP grafts composed of sequence between residues 29–52, 109–152 (IgGs 109–132, 119–142, and 129–152), and 159–231 (IgGs 159–182, 169–192, 172–196, 178–202, 189–212, 196–222, and 209–231), that did not bind to PrP in earlier immunoprecipitation experiments performed at pH 7.4 (*17*), also failed to react with abnormally folded PrP conformers in the acidic milieu (Supporting Information). Marked differences were seen however in the reactivity of a subset of PrP-grafted antibodies with PrP^{Sc} at pH 5.7. In particular, antibodies containing PrP sequence between residues 39 and 102 (IgGs 39–62, 49–72, 59–82, 69–92, and 79–102) that bound either very weakly or not at all with disease-associated forms of PrP at pH 7.4 interacted robustly with these molecular species in an identical assay performed at pH 5.7 (Figure 1). Binding was particularly strong with PrP grafts that contained octarepeat sequence. Therefore, to determine the ability of an individual octarepeat sequence to bind misfolded PrP at acidic pH, an antibody was prepared that contained a single PHGGGWGQ graft. This antibody did not recognize PrP at pH 7.4 but did PrP^{Sc} at pH 5.7 (Figure 1). Importantly, at pH 5.7, no binding was detected between any of the reactive motif-grafted IgGs and PrP^{C} present in the brains of normal, uninfected mice even when the grafted antibodies were employed at a concentration of 10 μg/mL (Figure 2).

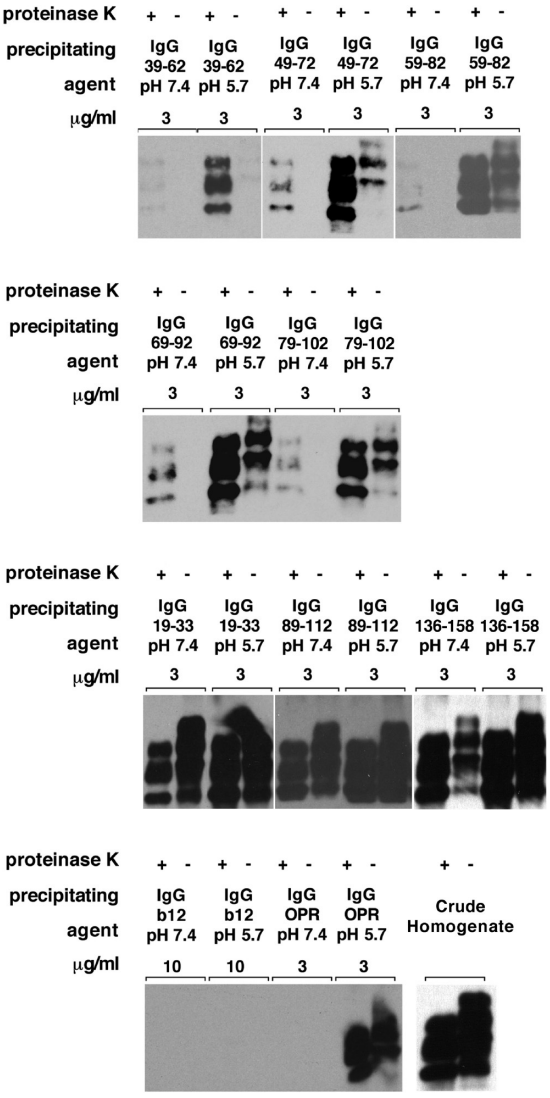

FIGURE 1: An acidic environment promotes reactivity of N-terminal PrP sequence with disease-associated PrP conformers. Mouse brain homogenates extracted with Triton X-100 were treated (+) or untreated (−) with proteinase K and then incubated with a panel of selected PrP-grafted antibodies. For each antibody, immunoprecipitation assay was carried out at neutral (pH 7.4) and acidic (pH 5.7) conditions. Antibody concentrations used in individual immunoprecipitation experiments are indicated. Grafted antibody was captured onto paramagnetic beads coupled to an anti-human IgG reagent, and any precipitated PrP was detected via Western blot by PrP-specific antibody 6H4.

Two of the motif-grafted antibodies with acquired reactivity for PrP^{Sc} at pH 5.7, IgGs 59–82 and OPR, were also tested under a panel of different pH conditions. Reactivity was measured at pH 7.4, 7.0, 6.6, 6.2, and 5.7, showing a dramatic increase at pH 6.2 and 5.7 (Figure 3). The diminished immunoprecipitation of PrP observed from non-protease-treated brain homogenate could be explained by the presence of endogenous PrP^{C}, which competes with the PrP-grafted antibody for PrP^{Sc} binding.

Point Mutations Indicate the 59–82 and Octapeptide PrP Peptide Grafts Require Positive Charge To Bind PrP^{Sc} at pH 5.7. At near neutral pH, peptides 59–82 and OPR possess a net charge of 0. However, below pH 6, in which protonation of the imidazole moiety positively charges the histidine side chain, peptide 59–82 and OPR acquired a net charge of +3 and +1, respectively (*35*). In the 59–82 peptide there are three histidine residues and in the single octapeptide one. To determine the contribution made by the positively charged amino acids to the 59–82 and octapeptide–PrP^{Sc} binding interaction at pH 5.7, modified 59–82 and octapeptide antibodies were prepared. These molecules contained histidine to alanine substitutions. In Figure 4 is shown that the replacement of the three histidine residues in 59–82 peptide and the one in the

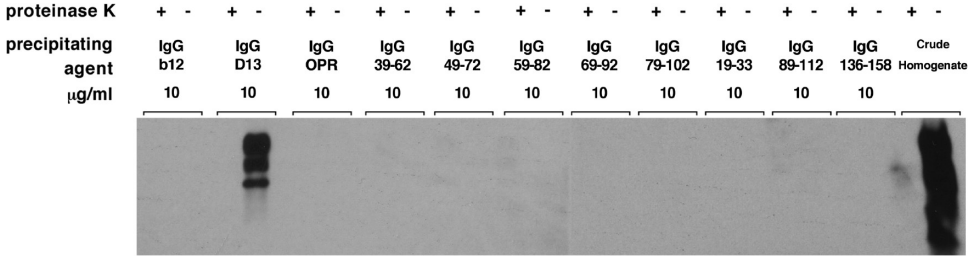

FIGURE 2: Under acidic conditions N-terminal PrP sequence does not recognize cellular PrP (PrP^{C}). The reactivity of PrP-grafted antibodies 39–62/49–72/59–82/69–92/79–102/octapeptide was tested in immunoprecipitation at pH 5.7 against brain homogenates prepared from non-PrP-infected mice as described in the legend of Figure 1. D13 antibody was used as positive control to immunoprecipitate PrP^{C}.

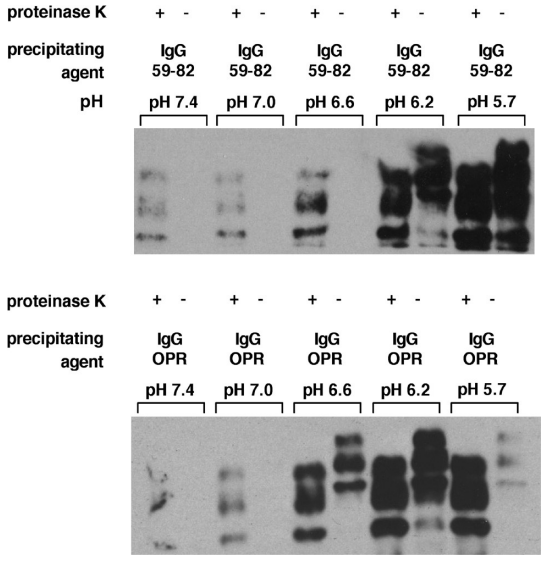

FIGURE 3: Reactivity of PrP-grafted antibodies 59−82 and OPR increase with acid pH. PrP-grafted antibodies 59−82 and OPR were evaluated in immunoprecipitation assay, as described in the legend of Figure 1, for the ability to recognize disease-associated PrP conformers present in brain homogenates prepared from scrapie-prion infected mice, under a panel of pH conditions.

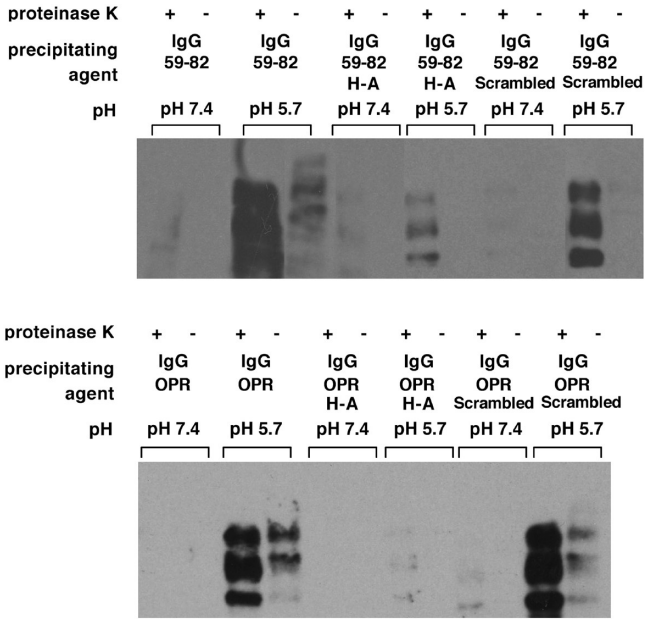

FIGURE 4: Histidine residues within the 59−82 and octapeptide sequence grafts play a crucial role in the recognition of disease-associated PrP conformers at pH 5.7. Histidine residues within the PrP59−82 and octapeptide sequence grafts were replaced by alanine residues. Additional antibodies were also generated in which the amino acidic sequences of the PrP59−82 and octapeptide sequence grafts were scrambled. Reactivity of the modified grafted antibodies with PrP^{Sc} and PrP27−30 present in brain homogenates prepared from scrapie-prion infected mice was evaluated, under neutral (pH 7.4) and acidic (pH 5.7) conditions, in an immunoprecipitation assay as described in the legend of Figure1.

octapeptide severely diminished (or almost abrogated) 59−82 and completely impaired octapeptide reactivity with PrP^{Sc} at pH 5.7. To understand if the interaction of 59−82 and octarepeat peptides with PrP^{Sc} at pH 5.7 was due exclusively to the presence of histidine residue, and not also to the specific amino acid sequences, antibody molecules containing scrambled sequences

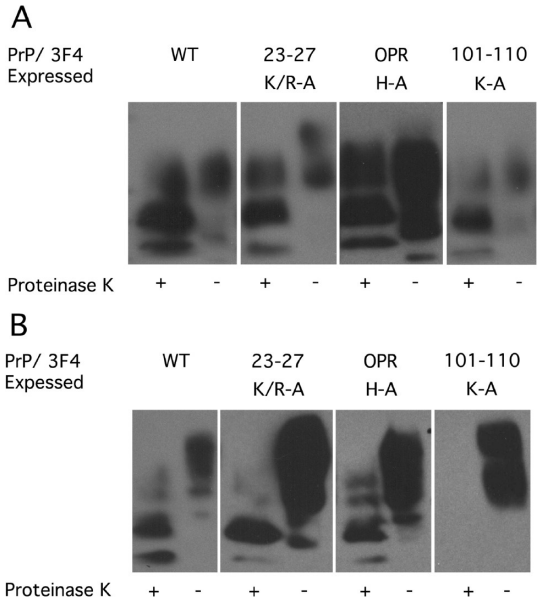

FIGURE 5: Modification of the octapeptide repeat of PrP does not impair conversion of PrP^{C} to PrP^{Sc} in ScN2a cells. Western blots of proteinase K treated and untreated ScN2a cell lysates transfected with 3F4-tagged WT-PrP and PrP mutants probed with antibody D18 for the detection of total PrP (A) or antibody 3F4 for the detection of transfected PrP (B). This figure was consolidated from multiple gels of a single experiment.

of the 59−82 and octapeptide were generated. As shown in Figure 4, most of the reactivity of scrambled 59−82 and octapeptide for PrP^{Sc} were retained. Interestingly, the 172−196 PrP graft contains two histidines, yet did not react with PrP at pH 7.4 or 5.7.

Histidine to Alanine Mutant PrP Is Not Impaired in Conversion to PrP^{Sc} in ScN2a Cells. With the use of motif-grafted antibodies, we determined that the octarepeat region of PrP specifically reacts with PrP^{Sc} in an acidic environment and that the substitution of octarepeat histidines with alanine abolished reactivity of the octarepeat region with PrP^{Sc}. To determine if these residues play a role in the conversion of PrP^{C} to PrP^{Sc}, we transfected a prion-infected N2a cell line with 3F4-tagged mutant PrP and assayed for proteinase K resistant PrP by Western blot. As shown in Figure 5, mutant PrP expressing histidine to alanine substitutions in the octarepeat is converted to the protease-resistant isoform as efficiently as wild type in N2a cells. Mutants 23−27 and 101−110 were previously reported and included here as controls.

The Protease-Resistant PrP That Reacts with the Histidines of the Octapeptide Repeat at pH 5.7 Can Seed the Conversion to PrP^{Sc} in Vitro. Because octarepeat histidine to alanine mutant PrP is not impaired in the ability to convert to PrP^{Sc} in N2a cells, we wanted to determine if the protease-resistant PrP that reacts with the octapeptide histidines at pH 5.7 is able to induce the conversion of normal PrP to a protease-resistant isoform in vitro. To do this, we seeded a protein misfolding cyclic amplification (PMCA) with the material precipitated at pH 5.7 from proteinase K treated RML infected mouse brain homogenate by PrP-grafted antibodies OPR, OPR H-A, and antibody b12 and at pH 7.4 by PrP-grafted antibody 89−112. As shown in Figure 6, material precipitated by 89−112 at pH 7.4 and OPR at pH 5.7 was able to induce the conversion of normal PrP to the protease-resistant form, whereas that by OPR H-A and b12 at pH 5.7 was not.

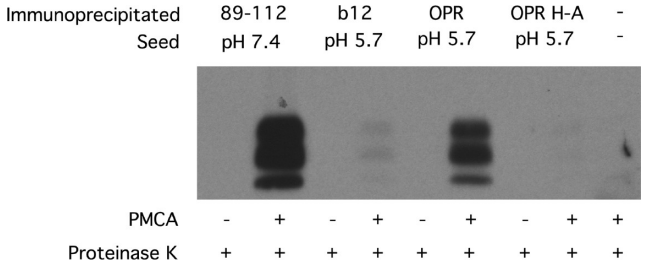

FIGURE 6: The proteinase K resistant PrP precipitated by the octapeptide repeat at pH 5.7 is able to seed conversion of PrPsen to PrPres by protein misfolding cyclic amplification. Proteinase K resistant PrP was captured onto PrP-grafted antibody coated beads at the pH indicated and used to seed a protein misfolding cyclic amplification. Seeded samples, subjected to sonication (+) or stored at −80 °C(−), and unseeded substrate were digested with proteinase K, and PrP was detected by Western blot using antibody D13.

DISCUSSION

In this work we describe the finding of the dramatic effect that the reduction of pH has upon the relative reactivity of specific PrP-grafted antibodies to PrP^{Sc}. In particular, at pH 5.7, the increase in reactivity observed for antibodies containing PrP sequence between residues 39 and 102 was very pronounced. Of potential importance, at pH 5.7, but not at pH 7.4, protonation of the imidazole moiety positively charges the histidine side chain, thereby effectively altering the net charge on a number of the PrP polypeptide grafts, including those containing octarepeat sequence (*35*). Given the role that positive charge clearly plays in PrP^{Sc} recognition by the 19−33 and 98−110 PrP grafts (*27*), the acquisition of a net positive charge following the transition to acidic conditions may be an important factor in the acquisition of PrP^{Sc} reactivity by some PrP grafts. However, the 172−196 PrP graft contains two histidine residues and yet does not recognize PrP^{Sc} at either pH 7.4 or pH 5.7. Thus a transition toward a net positive charge alone is in and of itself likely to be insufficient to explain the acquisition of PrP^{Sc} reactivity. An alternative explanation for our observations is that PrP^{Sc} itself may be modified by the switch to acidic pH. Such a modification, possibly manifesting through a change in aggregation state, or by subtle alterations in conformation, could expose to solvent otherwise hidden PrP^{C}-binding regions. Indeed, conceptually, the presence of multiple binding sites offers the possibility that a single PrP^{C} molecule, with its inherent conformational flexibility (*16*), may associate with multiple PrP^{Sc} molecules at a single time. As the cell internalizes the PrP^{C}−PrP^{Sc} complex, entering the endosomal pathway with the accompanying drop in pH, PrP^{C}−PrP^{Sc} couplings could then be stabilized or modified by additional interactions established via PrP^{C} sequence between residues 39−102.

The N-terminal domain of PrP has been shown to have a clear effect on the size and kinetics of PrP aggregates in vitro. In aggregation experiments performed in vitro at pH 4, it was shown that recombinant PrP 23−231 forms aggregates in two stages (*17*). The first stage consists of the depletion of monomeric PrP to form small aggregates. As the monomer becomes depleted, the process enters the second stage, and the small aggregates associate to form much larger aggregates. Interestingly, PrP 90−231 proceeds more slowly through the first stage and does not enter the second stage of aggregation. We have found two regions within the N-terminal domain of PrP that react with PrP^{Sc} under different pH conditions. They are 23−27 at both a neutral and acidic pH and octarepeat at an acidic pH.

While the N-terminal domain of PrP has been shown to not be required for prion infectivity, it has been shown to play an important role in both the stabilization of PrP^{C}−PrP^{Sc} interactions and aggregation in vitro and disease progression and pathology in vivo (*2*, *17*, *20*, *21*, *33*). PrP^{Sc} has also been shown to accumulate in the endosomal pathway (*24*, *31*, *32*). Our results show that the octarepeat region of PrP^{C} reacts with PrP^{Sc} at an acidic pH such as that of the endosomes.

On the basis of our evidence that pH can significantly modulate PrP^{C}−PrP^{Sc} binding interactions, we speculate that, in a cellular environment, the initial PrP^{C}−PrP^{Sc} binding, conversion, and further interaction of PrP^{Sc} with PrP^{C} may occur as a three-phase process. Initial contact between PrP^{C} and PrP^{Sc} and the conversion to a protease-resistant and infectious conformation may take place via one or more of the high-affinity neutral pH binding motifs we have previously identified. Following the conversion of PrP, the misfolded PrP may then enter the endosomal pathway, where the drop in pH causes the protonation of the histidines within the octarepeats of PrP^{C}, leading to further interaction with PrP^{Sc}.

ACKNOWLEDGMENT

We would like to thank R. Anthony Williamson for his help. We thank Dr. Michael B. Oldstone for the use of the PMCA equipment. Table of contents graphic by Christina Corbaci (ccorbaci@scripps.edu) and Justin Cruite.

SUPPORTING INFORMATION AVAILABLE

Western blot showing the PrP-grafted antibodies that did not react with PrP^{Sc} at pH 5.7. This material is available free of charge via the Internet at http://pubs.acs.org.

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