The AGAAAGA Palindrome in PrP Is Required to Generate a Productive PrPSC-PrPC Complex That Leads to Prion Propagation*

Received for publication, November 30, 2004, and in revised form, April 18, 2005
Published, JBC Papers in Press, May 25, 2005, DOI 10.1074/jbc.M413441200

Eric M. Norstrom‡ and James A. Mastriani§¶
From the §Committee on Neurobiology and ¶Department of Neurology, The University of Chicago, Chicago, Illinois 60637

The molecular hallmark of prion disease is the conversion of normal prion protein (PrPC) to an insoluble, proteinase K-resistant, pathogenic isoform (PrPSc). Once generated, PrPSc propagates by complexing with, and transferring its pathogenic conformation onto, PrPC. Defining the specific nature of this PrPSc-PrPC interaction is critical to understanding prion genesis. To begin to approach this question, we employed a prion-infected neuroblastoma cell line (ScN2a) combined with a heterologous yeast expression system to independently model PrPSc generation and propagation. We additionally applied fluorescence resonance energy transfer analysis to the latter to specifically study PrP-PrP interactions. In this report we focus on an N-terminal hydrophobic palindrome of PrP (112-AGAAAGA-119) thought to feature intimately in prion generation via an unclear mechanism. We found that, in contrast to wild type (wt) PrP, PrP lacking the palindrome (PrPΔ112–119) neither converted to PrPSc when expressed in ScN2a cells nor generated proteinase K-resistant PrP when expressed in yeast. Furthermore, PrPΔA112–119 was a dominant-negative inhibitor of wtPrP in ScN2a cells. Both wtPrP and PrPΔA112–119 were highly insoluble when expressed in yeast and produced distinct cytosolic aggregates when expressed as fluorescent fusion proteins (PrP::YFP). Although self-aggregation was evident, fluorescence resonance energy transfer studies in live yeast co-expressing PrPSc-like protein and PrPΔA112–119 indicated altered interaction properties. These results suggest that the palindrome is required, not only for the attainment of the PrPSc conformation but also to facilitate the proper association of PrPSc with PrPC to effect prion propagation.

The prion diseases, the best known of which include Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cows, result from the generation and propagation of a conformational variant (PrPSc) of the normal prion protein (PrPC), a predominantly brain-derived glycoprotein of unknown function (1). Once PrPSc is introduced or spontaneously generated in the brain, it can replicate by complexing with, and templating its conformation onto, additional PrP (2, 3). PrPSc is distinguished from PrPC in that it is insoluble in non-ionic detergents, displays a relative resistance to proteinase K (PK),1 and carries a significantly higher content of β-sheet structure (4). The molecular mechanisms underlying the conversion and replication of PrPSc are unknown.

A greater understanding of the specific regions of PrP that critically participate in the de novo generation and replication of PrPSc is a major goal of prion research, both in terms of understanding the well recognized species barrier to transmission and as a prelude to developing therapeutic interventions. While the C-terminal globular domain of PrPC is dominated by α-helical structure, the N-terminal region of the protein is unstructured by NMR (5). However, within the N-terminal half of PrP is the so-called “toxic peptide,” a highly conserved segment comprised of amino acids (aa) 105–125 (mouse sequence numbers). Synthetic peptides corresponding to this segment form fibrils in solution with β-sheet structure and are toxic to cultured mouse hippocampal cells (6–8), suggesting that this segment may feature in PrPSc-PrPC interactions. Within the toxic peptide, extending from aa 112–119 is a palindrome sequence (AGAAAGAGA), also known as the “hydrophobic core” (Fig. 1). This segment of PrP was found to be the most fibrilogenic motif within the toxic peptide (6), and later work showed that altering the sequence could modulate its structure and toxicity (9), spotlighting this minimal segment as an area that may feature intimately in the interaction of PrP molecules and their subsequent conversion to PrPSc. Consistent with this idea, larger peptides (aa 106–141) antagonized the in vitro conversion of PrP to the protease-resistant state in a cell-free conversion model, and this effect was dependent upon the inclusion of the AGAAGAGA sequence in the peptide (10). The hydrophobic core appears to undergo a profound conformational change upon conversion to PrPSc, since residues 90–120 are accessible to antibodies in PrPC yet not in PrPSc (11). In addition, antibodies directed against this region effectively “cured” a neuroblastoma cell line chronically infected with scrapie (12). Others have shown that deletion of a segment of PrP that overlaps the hydrophobic core produced a PrP that does not convert to PrPSc (13, 14).

While the above studies support the palindrome as critical to prion generation, direct mechanistic evidence for this is currently lacking. To provide such evidence and further define the role of the AGAAGAGA palindrome in prion processing, we applied a well documented neuroblastoma cell line chronically infected with prions (ScN2a) to model PrPSc replication and a heterologous yeast expression system (15) to model de novo PrPSc generation. We additionally applied live cell fluorescence resonance energy transfer (FRET) to the yeast model to assess the role of the palindrome in the assembly of PrP molecules. Our results suggest that the hydrophobic core is not required

1 The abbreviations used are: PK, proteinase K; aa, amino acids; PBS, phosphate-buffered saline; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ER, endoplasmic reticulum.
for PrP aggregation; however, it is critical, not only for the de novo generation of the specific PrPSc conformation but also for the proper association of PrPSc and PrPC molecules necessary for the conformational transfer. These results not only confirm the palindromic sequence as an essential component to prion genesis but also highlight the utility of this approach in resolving the specifics of prion conversion and propagation.

MATERIALS AND METHODS

Generation of PrP Mutants—The mouse PrP coding sequence was generated by PCR amplification and cloned into the pcDNA3 vector using standard molecular biological techniques. Deletion mutants were constructed from this template by PCR amplification with Pfu Turbo polymerase (Stratagene) using forward and reverse primers flanking the sequences to be deleted. The purified linear PCR product was then blunt end-ligated and transformed into XL-1 Blue supercompetent cells (Stratagene). All constructs to be expressed in mammalian cells were ligated into the pCMV vector (containing human promoters, a poly(A) addition signal, and a 2.2-kilobase fragment of the SV40 large-T antigen) and cloned into the mammalian expression vector pcDNA3. All constructs were confirmed by sequencing.

Yeast Expression System—The p4xGAL vectors contain a galactose-inducible expression element that is induced in the presence of galactose as the carbon source and tightly restricted when only glucose is present. In all experiments, we used the yeast strain W303 (MATa ade2–1 can1–100 his3–12,16 leu2–3,112 trpl–1 ura1–1) with a Pep4 genotype, which has reduced endogenous protease activity (kind gift of Susan Lindquist, MIT). For transformation, yeast colonies were picked from plates and grown overnight at 30 °C to mid log phase, washed, and incubated with the construct of interest plus polyethylene glycol, LiAc, and carrier DNA for 30 min at 4 °C followed by a 20-min heat shock at 42 °C and then spread onto selective plates made from glucose dropout media.

Solubility Assay—Yeast expressing the construct of interest were grown to mid-late log phase (A600 ~0.7), briefly spun down, washed in 10 mM EDTA, and digested with zymolyase 100T for 1–2 h at 30 °C. Spheroplasts were pelleted and lysed in 200 μl of 10% Sarkosyl in TEN buffer (40 mM Tris-Cl, pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl) with protease inhibitors for 5 min on ice. Insoluble PrP was found to effectively pellet following centrifugation at 16,000 × g for 30 min at 4 °C, which was used as a measure of insolubility. Following centrifugation, the supernatant was removed and the pellet washed with an additional 50 μl of 10% sarkosyl buffer, centrifuged again, and resuspended in 200 μl of 1% sulfobetaine in PBS. In some cases, the supernatant was subjected to centrifugation at 100,000 × g to ensure that all insoluble PrP was collected after the initial centrifugation and to check for any insoluble fraction of yellow fluorescent protein (YFP). For solubility in mammalian cell culture, transiently transfected cells were lysed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, and a portion was removed as the total fraction. The remaining lysate was spun at 16,000 × g, the supernatant was saved, and the pellet was resuspended in an equal volume of PBS with 1% sulfobetaine.

Protease Digestion Assay—For yeast lysates, PK digestions were performed on samples from the 16,000 × g pellet fraction of PrP or the supernatant fraction of YFP. Approximately 400 ng of total protein was digested with the indicated concentrations of PK (Fig. 3A) or with 40 μg/ml (all other figures). Protease inhibitors that are not known to interfere with PK digestion (0.5× chymostatin, pepstatin A, and aprotinin) were included in reactions for all figures. Control experiments indicated that chymostatin reduced the sensitivity of PrP to PK but did not induce protease resistance in control YFP or non-resistant PrP.

FRET—Fluorescence microscopy and photobleaching FRET experiments were performed with an Olympus IX51 microscope using appropriate Chroma filters. A Q-imaging Retiga EXI CCD and Metamorph software were used to capture images. PrP23–230::CFP donor was co-expressed with a candidate acceptor and yeast were scanned for the presence of both cyan fluorescent protein (CFP) and YFP signals using a 6% neutral density filter. Photos of yeast-containing aggregates were taken at 1-s intervals with 100 ms exposure times under constant illumination for 45 s. Image series were saved in stacks, and the illumination intensity profile of medium sized aggregates was analyzed across the time (Z) series using ImageJ (NIH) software. The decay of the donor fluorescence signal was fit with a single exponential decay curve, and an τ (τ) was generated using Igor Pro (Wavemetrics) software, using the equation Yn = Aexp(−(t/τ)X). The presence of both CFP and YFP signals for a given sample was confirmed before analysis. To control for differences in lamp intensity across data collection settings, which can alter the absolute bleach rate of samples, only two donor/acceptor pairs were measured at a time, and direct comparisons were made between these two data sets with relative comparisons made between pairs of data sets. Each pair of data sets is normalized to the set for which the lower τ value was obtained.

Confocal Immunofluorescence Microscopy—PrP constructs carrying the 3F4 epitope were transiently transfected into ScN2a cells grown on coverslips and slides using Lipofectamine 2000 reagent (Invitrogen), as per the manufacturer's instructions. To detect surface PrP after 36 h of expression, cells were washed three times in cold PBS and incubated for 1 h at 4 °C in PBS with 1% bovine serum albumin and the 3F4 monovalent antibody at a dilution of 1:50. Cells were washed and fixed in 4% paraformaldehyde for 30 min. After washing, cells were blocked with 5% milk, 1% bovine serum albumin in PBS for 30 min, then washed and incubated in Cy5-conjugated anti-mouse antibody at 1:100 dilution in PBS. Cells were then washed and covered in 80% glycerol in PBS. Cy5 signal was visualized using an Olympus IX70 with a Fluoview confocal module.

ScN2a Cell Line Conversion Assay—Transiently transfected cells from 60-mm dishes were lysed in 500 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) and cleared by centrifugation at 10,000 × g for 1 min. A total of 400 μl of the cleared lysate was digested with 10 μg/ml PK for 30 min at 37 °C and stopped with 2 mM phenylmethylsulfonyl fluoride, precipitated in methanol, resuspended in SDS-sample buffer, and submitted to SDS-PAGE and Western blotting, as described. For dominant-negative inhibition experiments, ScN2a cells were transfected with 4 μg each of plasmid DNA containing wtPrP carrying the 3F4 epitope and wt or mutant constructs without the 3F4 epitope. Lysis and digestion were carried out as described above.

Western Blots—Protein samples were precipitated in methanol for greater than 30 min at −20 °C. Following precipitation, samples were spun at 16,000 × g for 30 min at 4 °C and dried, and the pellets were resuspended in 2× sample buffer and loaded onto 16% (yeast) or 14%
Expression of recombinant PrP in a prion-infected mouse neuroblastoma cell line (ScN2a) (16) results in its conversion to PrPSc, provided it reaches the same cellular compartment as endogenously synthesized PrPSc and the two molecules interact in a way that supports the conformational transfer. As such, this cell line is useful to study the process of prion replication. To avoid the difficulties of others, such as specifically tracking recombinant mouse PrP in this mouse cell line (13), and using antibodies for PrP detection that may interfere with the conversion process (14, 17), we engineered the PrP constructs to include the hamster-specific 3F4 monoclonal antibody epitope (Met substitutions at residues 108 and 111), known not to interfere with the conversion process (17). ScN2a cells were transiently transfected with wtPrP or that lacking the hydrophobic core (Δ112-119) and, after 48 h of expression, the cells were assayed for newly synthesized PK-resistant PrP by Western blot. PrP is normally expressed as three molecular weight fractions, representing unglycosylated, monoglycosylated, and diglycosylated forms. PrPΔ112–119, which confirmed its localization to the plasma membrane (Fig. 2A), was not. In light of conversion process (17), ScN2a cells were transiently transfected with wtPrP or that lacking the hydrophobic core sequence (PrPΔ112–119) and, after 48 h of expression, the cells were assayed for newly synthesized PK-resistant PrPSc by Western blot. PrP is normally expressed as three molecular weight fractions, representing unglycosylated, monoglycosylated, and diglycosylated forms. PrPΔ112–119 expressed in these cells displayed similar levels and glycosylation profile as wtPrP, suggesting normal transit through the Golgi apparatus (Fig. 2A). However, whereas wtPrP was efficiently converted to PK resistant PrP, PrPΔ112–119 was not. In light of evidence that PrPC must traffic to the cell surface before it is made available to complex with, and be converted to, PrPSc by Western blot, PrPSc is not converted to PrPSc in the absence of the palindrome, PrP undergoes normal processing and trafficking (ScN2a lysates) SDS-polyacrylamide gels. Following electrophoresis, samples were transferred onto polyvinylidene difluoride membranes. These were washed in TBST (20 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.6) and blocked for 1 h with 5% milk, washed, and incubated in TBST with 1% milk containing primary antibodies at a 1:5000 (R1) or 1:3000 (3F4) dilution for 3 h at room temperature or overnight at 4 °C. Following a wash, the membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and detected using ECL plus (Amersham Biosciences).

Densitometry—For each Western blot on which densitometry measurements were to be taken, multiple film exposures of increasing length were performed. Densitometry measurements were obtained from the “total” sample for each exposure time, and a curve was generated from the results. For reported experiments, exposure times were used in which data from the total sample were in the linear range of measurements. Films were scanned using an Epson transparency scanner, and densitometry was performed using UN-scan-it (Silk Scientific) software.

RESULTS

Expression of recombinant PrP in a prion-infected mouse neuroblastoma cell line (ScN2a) (16) results in its conversion to PrPSc, provided it reaches the same cellular compartment as endogenously synthesized PrPSc and the two molecules interact in a way that supports the conformational transfer. As such, this cell line is useful to study the process of prion replication. To avoid the difficulties of others, such as specifically tracking recombinant mouse PrP in this mouse cell line (13), and using antibodies for PrP detection that may interfere with the conversion process (14, 17), we engineered the PrP constructs to include the hamster-specific 3F4 monoclonal antibody epitope (Met substitutions at residues 108 and 111), known not to interfere with the conversion process (17). ScN2a cells were transiently transfected with wtPrP or that lacking the hydrophobic core sequence (PrPΔ112–119) and, after 48 h of expression, the cells were assayed for newly synthesized PK-resistant PrPSc by Western blot. PrPSc is not converted to PrPSc in the absence of the palindrome, PrP undergoes normal processing and trafficking yet is not converted to PrPSc.

The lack of conversion of PrPΔ112–119 to PrPSc was considered to result either from poor or non-productive interaction with endogenous PrPSc or an inability to attain the PrPSc.
conformation. To address these possibilities, we first tested the ability of PrPΔ112–119 to inhibit the conversion of wtPrP in a dominant-negative fashion (20). Such a feature would suggest interaction with PrPSc. ScN2a cells were co-transfected with equal amounts of PrPΔ112–119 PrP and wtPrP constructs, the latter of which carried the 3F4 antibody epitope. After 48 h of expression, PK-digested lysates were analyzed by Western blot, using antibody 3F4, to selectively detect nascent wtPrPSc. When these results are compared with those of lysates co-expressing wtPrP(3F4) and untagged wtPrP, a clear inhibition of wild type conversion by PrPΔ112–119 is evident (Fig. 2C, lanes 1 and 2). As a positive control, wtPrP(3F4) was co-expressed with untagged PrP carrying the Q218K mutation, a known dominant-negative inhibitor of wild type prion conversion (Fig. 2C, lane 3). Here again, conversion of wtPrP(3F4) was significantly inhibited in the presence of the mutant protein.

We interpret the above results to indicate that PrPΔ112–119 is forming a non-productive association with endogenous PrPSc and in so doing interferes with conversion of wtPrPSc. In support of this, we compared the solubility of wtPrP and PrPΔ112–119, following expression in ScN2a cells. Both were detected within the 16,000 × g insoluble fraction of detergent-lysed ScN2a cells, although the fraction of PrPΔ112–119 was 30% lower than that of wtPrP (Fig. 2, D and E). In contrast, the fraction of insoluble PrP was significantly lower when these were expressed in cells, which do not endogenously express PrP (COS-7) (Fig. 2, D and E). These data support the association of PrPΔ112–119 with PrPSc.

We next sought to investigate the de novo generation of PrPSc and more directly explore the alteration in interaction properties of wild type and deletion mutant PrPs, as suggested by the ScN2a cell experiments. To accomplish this, we employed a heterologous yeast expression system that supports the generation of a PrPSc-like protein (15), as defined by its insolubility and PK resistance. Mature PrP excluding the ER and glycosylphosphatidylinositol anchor signal sequences (PrP23–230) was expressed into the yeast cytosol under the control of the GAL1-inducible promoter for 16 h, at which time cells were detergent-lysed and PrP pelleted by centrifugation at 16,000 × g. Since this protein is expressed into the cytosol, ER- and Golgi-related glycosylation does not occur, resulting in a single unglycosylated band of ~25 kDa. As expected, insoluble wtPrP was resistant to proteolysis in a dose-dependent manner (Fig. 3A). The predominant PK-resistant fraction was ~20 kDa, which compares well with that detected in the brain of subjects with prion disease and ScN2a cells. To confirm that overexpression of any protein into the cytosol of yeast does not invariably develop PK resistance, we expressed YFP under similar conditions of expression and recovered no protease resistance, despite high levels of expression and challenge with the lowest concentration of PK (Fig. 3A). This demonstrated selectivity suggested that the yeast model would be useful to study the molecular substrates required for PrPSc generation as a complement to the ScN2a model of PrPSc replication.

We next applied the yeast expression system to study the role of the palindrome in the de novo generation of PrPSc. PrP23–230, excluding an 105–125, was initially expressed in the yeast cytosol, since this segment has received considerable attention as the toxic unit of PrP. In contrast to wtPrP (Fig. 3B, lanes 1 and 2), PrPΔ105–125 was highly susceptible to PK (Fig. 3B, lanes 3 and 4), displaying no resistance at the lowest concentration. Reduction of the deleted region to exclude only the AGAAAAGA palindrome (PrPΔ112–119) did not re-establish the PK-resistant phenotype (Fig. 3B, lanes 5 and 6). However, when a significantly redacted PrP, containing a large deletion lying N-terminal to both the palindrome segment and the PK-resistant core of PrPSc (PrPΔ1–89), was expressed in the yeast cytosol, the pattern of PK resistance was similar to that of full-length wtPrP (Fig. 3C), supporting a deletion-specific effect and a critical role for the palindrome in the generation of the PrPSc-like conformation.

We questioned whether the inability of deletion mutants to generate the PrPSc conformation resulted from their failure to self-assemble. If the hydrophobic core is solely responsible for PrP self-associations, we reasoned that PrPΔ112–119 should be soluble when expressed in the yeast cytosol. We therefore prepared lysates from yeast expressing wtPrP, PrPΔ112–119, PrPΔ1–89, and YFP, using 10% Sarkosyl/TEN buffer, and separated soluble and insoluble fractions by centrifugation at 16,000 × g. The fraction of soluble PrP and YFP was determined by densitometric analysis of the Western blot signal. As expected, wtPrP and PrPΔ1–89 were highly insoluble, with only ~10% recoverable from the supernatant (Fig. 4). In contrast, 100% of expressed YFP remained in the soluble fraction,
even following centrifugation at 100,000 \times g (Fig. 4 and data not shown), confirming that insolubility is a specific feature of PrP and not a property of overexpression into the yeast cytosol. PrPΔ112–119 displayed only marginally greater solubility than wtPrP, suggesting that insolubility is not solely dependent on the AGAAAAGA palindrome.

We next attempted to visually confirm that wtPrP and/or PrPΔ112–119 aggregate within the yeast cytosol, and if so, were the aggregates distinguishable from each other. To do so, we prepared wt and PrPΔ112–119 constructs on the PrP23–230 backbone with YFP linked to the C-terminal end. These fluorescent fusion proteins were individually expressed into the yeast cytosol for 16 h following transformation, at which time yeast were visualized directly by fluorescence microscopy. In cells expressing only YFP, a diffusely uniform signal was evident throughout the cytosol (Fig. 5A, top), whereas in those expressing the wtPrP::YFP fusion protein, distinct cytosolic aggregates were evident, in addition to a low level of diffuse background signal, presumably representing either soluble PrP not yet aggregated or proto-aggregates that are beyond the limit of resolution of light microscopy (Fig. 5A, middle row). The cytosolic inclusions were visualized as early as 5 h after induction of expression and were maximum by 16 h. As predicted from the solubility data, PrPΔ112–119::YFP produced visible aggregates that were grossly indistinguishable from wtPrP::YFP (Fig. 5A, bottom row). Thus, although the AGAAAAGA palindrome appears to be essential for generation of the PrPSc conformation, it does not appear to be essential for the bulk aggregation/association of PrP molecules.

In light of the PK sensitivity of yeast-expressed PrPΔ112–119, its lack of conversion to PrPSc following expression in ScN2a cells, and its dominant-negative inhibition behavior, we questioned whether the intermolecular association of PrPΔ112–119 with PrPSc differs fundamentally from that of wtPrPSc. To investigate the interactive properties of PrP in the cytosol of live yeast, we first co-expressed PrP fusion proteins tagged with either CFP or YFP and visualized their behavior using standard fluorescent microscopy. PrP::CFP co-expressed with YFP resulted in aggregated PrP fusion molecules and soluble YFP (Fig. 5B, top). In virtually 100% of cases where both proteins were visible within the same yeast cell, wtPrP::CFP colocalized with wtPrP::YFP, as expected (Fig. 5B, middle row). In addition, when wtPrP::CFP and PrPΔ112–119::YFP were co-expressed, these also appeared to co-localize (Fig. 5B, bottom row). While these results suggest that the two proteins were associating to form heterologous aggregates, it was also considered that homologous molecules were self-associating in an ordered way prior to their bulk coalescence into large disordered heterologous aggregates that were visible at the level of fluorescence microscopy.

To better determine the specific nature of PrP associations, we applied the technique of donor photobleaching FRET to the yeast expression system. FRET is a powerful tool for investigating protein interactions that exploits the non-radiative transfer of energy from an excited-state donor fluorophore to an acceptor fluorophore (21, 22). When FRET occurs, a decrease in the bleaching rate of the donor molecule is detected, because energy that would normally be used for local oxidation reactions is transferred to the acceptor molecule instead. By measuring the decay rate of donor fluorescence in yeast aggregates, comparisons can be made as to the relative interactions of different donor/acceptor pairs. The emission/ excitation spectra of CFP (donor) and YFP (acceptor) fluorescent proteins, used in the construction of the PrP fusion proteins, are well suited for use in FRET microscopy in live cells (23, 24). To first determine whether FRET between PrP molecules would be measurable in this system, we co-expressed PrP::CFP with PrP::YFP in yeast and measured the decay ($\tau_{bl}$) of the donor (CFP) fluorescence signal upon bleaching under constant illumination. This measurement was compared with that of PrP::CFP co-expressed with YFP, since YFP does not aggregate in the yeast and is therefore predicted not to specifically interact with PrP. As expected, the donor $\tau_{bl}$ is significantly ($p < 0.001$) extended when the donor/acceptor pair is PrP::CFP/PrP::YFP, compared with PrP::CFP/YFP, confirming that donor and acceptor PrP fusion proteins undergo specific interactions within the yeast cytosol and that this interaction is measurable (Fig. 6, A and B).

We next applied this technique to determine whether PrPΔ112–119::YFP and PrP::CFP specifically associate. Using the donor/acceptor pair PrP::CFP/YFP as a non-FRET pair for comparison, we found that, despite the apparent co-aggregation by fluorescent light microscopy, when PrPΔ112–119::YFP and PrP::CFP were co-expressed and analyzed for FRET, the $\tau_{bl}$ value for this pair did not differ significantly from the non-FRET pair (Fig. 6B). However, a slight, but non-significant, increase in the decay rate, possibly due to molecular crowding by nonspecific disordered aggregation, was noted. As a confirmatory study, we directly compared the FRET efficiencies of PrP::CFP/PrPΔ112–119::YFP with the positive FRET pair, PrP::CFP/PrP::YFP, and found that the $\tau_{el}$ for the PrP::CFP/PrPΔ112–119::YFP pair was significantly less ($p < 0.001$) than that measured for the positive FRET pair. Last, we compared the $\tau_{el}$ for the self-association of deletion mutant PrPs (PrPΔ112–119::CFP/PrPΔ112–119::YFP) with the self-as-
association of wtPrPs (PrP::CFP/PrP::YFP) to determine whether the associative properties of PrPΔ112–119 are inherently different. Interestingly, the wtPrP interaction displayed a significantly greater (p < 0.001) FRET response than the deletion mutants, suggesting that PrPΔ112–119 undergoes a qualitatively different interaction with wtPrP and itself. These data, together with those of the yeast expression and ScN2a dominant-negative studies, support an essential role for the palindrome segment of PrP, not only in the attainment of the PrPSc conformation but also for the precise association of PrP molecules necessary for the subsequent conversion to PrPSc.

**DISCUSSION**

**Domains Involved in PrPSc Generation**—The pathogenesis of prion disease is a two-step process. First, the infectious prion (i.e. PrPSc) must be generated, either by spontaneous conversion of endogenous PrPC or by the exogenous introduction of PrPSc, such as with the ingestion of bovine spongiform encephalopathy-tainted beef. Once present within the host, PrPSc replicates by complexing with endogenous PrPC, onto which it templates the PrPSc conformation. In the absence of the three-dimensional structure of the N terminus of PrPSc, a precise model of PrPSc and the interface of the PrPSc-PrPC complex required to achieve the conformational transfer is not known. Defining the regional domains or residues within PrP that are essential for the de novo generation of PrPSc and/or the binding of PrPSc to PrPC is crucial, not only to better understand the process of prion biogenesis but also to clarify other features of prion biology, such as the species barrier and host susceptibility, in addition to assisting in the development of therapeutic strategies.
Here we focused on an 8-amino acid AGAAAAGA palindrome within the N-terminal region of PrP, previously implicated in prion genesis. Several lines of indirect evidence suggest that, although unstructured in solution, the N-terminal region of PrP may be intimately involved in the generation of PrPSc (7, 11, 25), and predicted models of PrPSc support extension of β-sheet structure into this area (26). We first studied the potential for PrPΔ112–119 to be converted to PK-resistant PrP during expression in ScN2a cells, a model of PrPSc propagation. We found that despite similar levels of expression and trafficking as wtPrP, PrPΔ112–119 was not converted to PK resistant PrP in this system. This agrees well with an earlier report (13) in which PrP, carrying a deletion that overlaps the palindrome (aa 114–121), was not converted to PrPSc in a different cell line chronically infected with prions (Sc−MNB cells). Additionally, PrPΔ112–119 inhibited conversion of wtPrP to PrPSc in a dominant-negative manner, suggesting that it binds to, but is not converted by, endogenous PrPSc, a result supported by the detection of PrPΔ112–119 in the insoluble fraction of ScN2a cell lysates.

The Yeast Model of Prion Biogenesis—The principal biological models used for the study of prion disease, such as rodent transmission bioassays and cell lines chronically infected with prions, do not directly address the process of de novo generation of PrPSc. The directed expression of wtPrP into the yeast cytosol was previously shown to support the de novo development of a PrPSc-like protein, as defined by the acquisition of insolubility and PK resistance (27). We tested the usefulness of this model to define the intramolecular substrates of PrP required for the de novo generation of PrPSc. First, we confirmed that the properties of aggregation, insolubility, and PK resistance are specific to PrP, since YFP expression in yeast did not display similar properties. We then found that selective deletion of either the 105–125 segment, or the AGAAAAGA palindrome contained within, prevented the generation of PK resistant PrP, suggesting that this property is not an invariant feature of PrP expression in this system, but rather it is critically dependent on the presence of specific “prion domains.” This lack of PK resistance supports the palindromic as one such segment necessary for generating the PrPSc conformation.

The N-terminal deletions did not measurably alter the solubility of PrP nor did they prevent the formation of visible aggregates when expressed as PrP::YFP fusion proteins. This finding not only indicates that self-association of PrP is not directed solely by the palindromic, but it also demonstrates that aggregation and insolubility of PrP are not firmly linked to PK resistance. Since PrPΔ1–89 also forms PK-resistant protein in this system, the secondary site(s) of association to explain the persistent aggregation of these redacted PrPs are likely C-terminal to the palindromic. Prior work, using a cell-free PrPSc replication assay of hamster PrP, found the addition of excess C-terminal peptides (aa 166–179 and 200–223) to the reaction mixture produced variable degrees of inhibition of PrPSc generation (28) and antibodies directed to C-terminal regions similarly abrogated PrPSc production in ScN2a cells (25). The delineation of the specific sites of involvement is easily approachable in this yeast system and is currently under study.

Live Cell FRET Defines Specific PrP Associations in Yeast—A distinct advantage of the yeast system over that of mammalian cell culture in the study of PrP associations is that a variety of confounding variables, such as protein trafficking and compartmental co-localization, are avoided, leaving only the interactive properties of PrP molecules as the primary determinants for association. As such, this provides an ideal system for the application of live cell FRET. Using a variation of FRET that involves photobleaching of the donor molecule and measurement of the decay rate of the excitation emission in the presence and absence of putative acceptors, we found this technique to be specific and sensitive. Whereas FRET was clearly evident between wtPrP::CFP and wtPrP::YFP, suggesting a specific association of the two, co-expression of PrPΔ112–119::YFP with either wtPrP::CFP or PrPΔ112–119::CFP, showed decay rates that were not significantly different from those measured during co-expression of a non-FRET pair. While this would suggest that these are non-associating proteins, the formation of visible aggregates of PrPΔ112–119::YFP when expressed in yeast suggest an alternate explanation. A caveat regarding the interpretation of FRET data is that FRET only occurs when the donor and acceptor molecules are within ~10 nm of each other, which allows for the situation in which there is a significant shift in the orientation of interacting proteins, resulting in the absence of FRET. Hence, the combination of visible aggregates and insolubility of PrPΔ112–119 expressed in yeast support such a hypothesis.

When the FRET results are combined with the results of ScN2a cell culture experiments, a model in which PrPSc and PrPSc associate at multiple, non-contiguous sites during the formation of the PrPSc-PrPSc complex can be envisioned. In this model, a primary site of association is the palindromic segment of the hydrophobic core, the deletion of which renders the molecule incapable of generating the PrPSc conformation and reveals, based on its ability to aggregate in yeast, association sites within the C-terminal portion of PrP. Since PrPΔ112–119 is not competent to spontaneously generate PrPSc in the yeast cytosol, the C-terminal association sites are presumed to be insufficient to generate PrPSc. However, the C-terminal sites, along with the palindromic, may function to properly align PrPSc with PrPSc to facilitate the conformational transfer necessary for prion replication. When either site is absent, a proper PrPSc-PrPSc interaction cannot take place, resulting in an altered association that does not support conversion and furthermore inhibits conversion of PrPSc to PrPSc in a dominant-negative fashion by occupying sites of prion extension.

The C-terminal areas involved in this interaction may be identical to those that have been previously considered “Protein X” binding sites. Protein X was first hypothesized as a host-specific co-factor that facilitates prion conversion, as a way to explain certain properties of prion genesis, such as the inability of certain prion strains to cross species, and dominant-negative inhibition of a competing PrP (29). However, our data suggest that Protein X, in its current conception, may not need to be invoked to explain such behavior. For instance, while the Q218K mutation is proposed to render PrPSc with greater affinity for Protein X thereby effectively sequestering it from the conversion reaction (29), we find that either removal of the palindromic or introduction of the Q218K mutation produces dominant-negative inhibition of prion propagation in ScN2a cells. Furthermore, others have reported that a double mutant containing the Q218K mutation and an N-terminal deletion very similar to ours (Δ107–120) does not produce dominant-negative inhibition (20). This result, when combined with our data, is consistent with the model we propose that predicts both sites are required for the proper association and alignment of PrPSc-PrPSc to effect the conformational transfer, while the absence of either site leads to a non-functional association that acts as a dominant-negative inhibitor of propagation. When both sites are lacking, interaction is prevented altogether, which leads to a loss of dominant-negative inhibition.

In summary, we employed a heterologous yeast expression system to study the intramolecular determinants of the de novo generation of PrPSc, as a compliment to the ScN2a model of PrPSc propagation. Our results support the necessity of the
AGAAAAGA palindrome, not only for the attainment of the PrPSc conformation but also for the formation of a productive PrPSc-PrPSc complex that results in the propagation of PrPSc. This first application of live cell FRET in yeast supports this as a promising approach to understand the nature of prion generation and replication. Identification of sites and regions of the protein required for conversion will also assist in the production of site-specific small molecules, antibodies, or peptides that could act as specific inhibitors of prion generation.

Acknowledgments—We thank Susan Lindquist and Jiyan Ma for their guidance in developing the yeast expression system.

REFERENCES

1. Mastrianni, J. A., and Roos, R. P. (2000) Semin. Neurol. 20, 337–352
2. Prusiner, S. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13363–13368
3. Caughey, B. (2001) Trends Biochem. Sci. 26, 235–242
4. Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E., and Prusiner, S. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10962–10966
5. Riek, R., Horнемann, S., Wider, G., Billette, M., Glockshuber, R., and Wuthrich, K. (1996) Nature 382, 180–182
6. Gasset, M., Baldwin, M. A., Lloyd, D. H., Gabriel, J. M., Holtzman, D. M., Cohen, F. E., Fletterick, R., and Prusiner, S. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10940–10944
7. Furloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O., and Tagliavini, F. (1993) Nature 362, 543–546
8. Brown, D. R., Herms, J., and Kretzschmar, H. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6597–6603
9. Muramoto, T., Scott, M., Cohen, F. E., and Prusiner, S. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1557–1565
10. Butler, D. A., Scott, M. R., Bockman, J. M., Taraboulos, A., Hsiao, K. K., Kingsbury, D. T., and Prusiner, S. B. (1991) J. Virol. 65, 6597–6603
11. Prusiner, S. B., and Burton, D. R. (1997) J. Mol. Biol. 273, 614–622
12. White, A. R., Enever, P., Tayebi, M., Manchon, R., Linehan, J., Brandner, S., Anstee, D., Collinge, J., and Hawke, S. (2003) Nature 422, 80–83
13. Holscher, C., Delius, H., and Burkle, A. (1998) J. Virol. 72, 1153–1159
14. Muramoto, T., Scott, M., Cohen, F. E., and Prusiner, S. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15457–15462
15. Ma, J., and Lindquist, S. (2002) Science 296, 1785–1788
16. Butler, D. A., Scott, M. R., Bockman, J. M., Borchelt, D. R., Taraboulos, A., Hsiao, K. K., Kingsbury, D. T., and Prusiner, S. B. (1996) J. Virol. 62, 1558–1564
17. Scott, M. R., Kohler, R., F., and Prusiner, S. B. (1992) Protein Sci. 1, 886–897
18. Caughey, B., Raymond, G. J., Ernst, D., Race, B., Robertson, M. N., Miyazawa, M., Mori, S., Evans, L. H., Hayes, S. F., Chesebro, B., Caughey, B. W., Dung, A., Bhat, K. S., Caughey, W. S., and Neary, K. (1991) J. Virol. 65, 597–6003
19. Kaneko, K., Vey, M., Scott, M., Pilluhn, S., Cohen, F. E., and Prusiner, S. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2333–2338
20. Zulianello, L., Kaneko, K., Scott, M., Erpel, S., Han, D., Cohen, F. E., and Prusiner, S. B. (2000) J. Virol. 74, 4351–4360
21. Kaneworthy, A. K. (2001) Methods (San Diego, CA) 24, 289–296
22. Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. (2001) Nat. Rev. Mol. Cell Biol. 2, 444–456
23. Majoul, I., Straub, M., Hell, S. W., Duden, R., and Soling, H. D. (2001) Dev. Cell 1, 139–153
24. Llopis, J., Westin, S., Ricote, M., Wang, Z., Cho, C. Y., Kurkowia, R., Mullen, T. M., Rose, D. W., Rosenfeld, M. G., Tsien, R. Y., Glass, C. K., and Wang, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4363–4368
25. Peretz, D., Williamson, R. A., Kaneko, K., Vergara, J., Leclerc, E., Schmitt-Ulms, G., Mehlhorn, I. R., Legname, G., Wormald, M. R., Rudd, P. M., Dwek, R. A., Burton, D. R., and Prusiner, S. B. (2001) Nature 412, 739–743
26. Wille, H., Michelitsch, M. D., Guenebaut, V., Suppatapone, S., Serban, A., Cohen, F. E., Agard, D. A., and Prusiner, S. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3563–3568
27. Ma, J., and Lindquist, S. (1999) Nat. Cell Biol. 1, 358–361
28. Horiiuchi, M., Barron, G. S., Xiong, L. W., and Caughey, B. (2001) J. Biol. Chem. 276, 15489–15497
29. Kaneko, K., Zulianello, L., Scott, M., Cooper, C. M., Wallace, A. C., James, T. L., Cohen, F. E., and Prusiner, S. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10069–10074
The AGAAAAGA Palindrome in PrP Is Required to Generate a Productive PrP<sup>Sc</sup>-PrP<sup>C</sup> Complex That Leads to Prion Propagation

Eric M. Norstrom and James A. Matrianni

J. Biol. Chem. 2005, 280:27236-27243.  
doi: 10.1074/jbc.M413441200 originally published online May 25, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413441200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/280/29/27236.full.html#ref-list-1