Strain-specific propagation of PrPSc properties into baculovirus-expressed hamster PrPC

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The conversion of the cellular isoform of the prion protein (PrP\textsuperscript{C}) to the abnormal disease-associated isoform (PrP\textsuperscript{Sc}) has been simulated in cell-free conversion reactions in which PrP\textsuperscript{Sc}-enriched preparations induce the conformational transition of PrP\textsuperscript{C} into protease-resistant PrP (PrP-res). We explored the utility of recombinant hamster (Ha)PrP\textsuperscript{C} purified from baculovirus-infected insect cells (bacHaPrP\textsuperscript{C}) as a replacement for mammalian-derived HaPrP\textsuperscript{C} in the conversion reactions. Protease-resistant recombinant HaPrP was generated after incubation of \textsuperscript{35}S-bacHaPrP\textsuperscript{C} with PrP\textsuperscript{Sc}-enriched preparations. Moreover strain-specific PrP-res was also reproduced using insect-cell derived HaPrP\textsuperscript{C} and PrP\textsuperscript{Sc} from two different strains of hamster-adapted transmissible mink encephalopathy, designated hyper (HY) and drowsy (DY). Two strain-mediated properties were tested: (i) molecular mass of the protease-digested products and (ii) relative resistance to proteinase K (PK) digestion. Similar to \textit{in vivo} generation of PrP\textsuperscript{HY} and PrP\textsuperscript{DY}, the converted products selectively reproduced both characteristics, with the DY conversion product being smaller in size and less resistant to PK digestion than the HY product. These data demonstrate that non-mammalian sources of recombinant HaPrP can be converted into PK-resistant form and that strain-mediated properties can be transmitted into the newly formed PrP-res.

Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of unusual neurodegenerative diseases that include Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Sträussler–Scheinker syndrome and kuru in humans, as well as scrapie in sheep, transmissible mink encephalopathy (TME) and bovine spongiform encephalopathy. These diseases are characterized by the accumulation of abnormal aggregated isoforms of the prion protein (PrP\textsuperscript{Sc}) in affected animals. The crucial pathogenic event in TSE diseases appears to be the conformational transition of the normal cellular PrP (PrP\textsuperscript{C}) into PrP\textsuperscript{Sc}. PrP\textsuperscript{Sc} forms insoluble aggregates that are protease-resistant and that have higher \(\beta\)-sheet content than PrP\textsuperscript{C}. Although the biochemical basis for the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} remains unknown, direct PrP\textsuperscript{C}–PrP\textsuperscript{Sc} interactions appear to be involved (Caughey & Chesebro, 1997). Current models for PrP\textsuperscript{Sc} replication include template-assisted conversion (Prusiner, 1991), in which the conformational transition of PrP\textsuperscript{C} is induced by a monomeric PrP\textsuperscript{Sc} through a cycle of unfolding and refolding reactions, and nucleation-dependent polymerization (Jarrett & Lansbury, 1993), where infectious PrP\textsuperscript{Sc} is an ordered aggregate that acts as a nucleant. PrP\textsuperscript{C}, upon binding to the seed, acquires the conformation of the PrP\textsuperscript{Sc} polymer.

According to the conformational hypothesis of strain variation, distinct PrP\textsuperscript{Sc} infectious conformers are able to impart their particular properties onto host PrP\textsuperscript{C} (Cohen & Prusiner, 1998). PrP\textsuperscript{Sc} conformations are an important component of TSE strain diversity (Parchi \textit{et al}., 1996; Collinge \textit{et al}., 1996; Telling \textit{et al}., 1996; Hill \textit{et al}., 1997; Rubenstein \textit{et al}., 1998; Safar \textit{et al}., 1998; Aucouturier \textit{et al}., 1999; Kuczius & Groshup, 1999; Bartz \textit{et al}., 2000), suggesting that different biochemical properties of PrP\textsuperscript{Sc} account for the strain phenotypes observed \textit{in vivo}.

The \textit{in vitro} assay for the conversion reaction of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, in the presence of exogenous PrP\textsuperscript{Sc} (Kocisko \textit{et al}., 1994), reproduces several biological properties of the \textit{in vivo} TSE infection such as strain-diversity, species-specificity and PrP polymorphism barrier phenomena (Bessen \textit{et al}., 1995; Kocisko \textit{et al}., 1995; Raymond \textit{et al}., 1997; Bossers \textit{et al}., 2000).
In the present study, recombinant HaPrP<sup>C</sup>, expressed in a non-mammalian system (baculovirus-infected insect cells), was readily converted to a proteinase K (PK)-resistant form in a cell-free system. These conversion reactions were performed using PrP<sup>Sc</sup> purified from two strains of hamster-adapted TME in combination with 35S-labelled recombinant hamster (Ha)PrP<sup>C</sup> (35S-bacHaPrP<sup>P</sup>). In addition to the distinguishing PK cleavage site (Bessen et al., 1995), another strain-specific property of hamster-adapted TME, differential PK resistance, was found to be transmitted to the newly converted 35S-bacHaPrP<sup>P</sup>-res. These studies show that recombinant PrP from a non-mammalian source can be converted into PrP-res forms, whose properties mimic in vivo PrP<sup>Sc</sup>.

**Methods**

**Plasmid construction.** A 781 bp DNA fragment containing the entire 254 amino acid hamster PrP<sup>C</sup> open reading frame was amplified from hamster genomic DNA using the primers Sph1 (−14 to +33: 5' GCAGATCAGCCATGGGAACCTTACTGCTTGGCACTC 3') and Shp2 (+734 to +767: 5' CCAATCCCCATGAGAAAAATGGA 3'). The PCR fragment was ligated into the baculovirus transfer vector pBlueBacIII (Invitrogen) using Bgl II and Pst I unique restriction sites, producing the recombinant vector pBlueBacIII-HaPrP<sup>P</sup>.

**Cell and virus culture.** Cultures of Spodoptera frugiperda (Sf21 cells) were co-transfected with the pBlueBacIII-HaPrP<sup>P</sup> construct and linearized baculovirus DNA using the Invitrogen Bac-N-Blue transfection kit. Seventy-four purified plaques of the recombinant virus were used to infect Sf21 cultures and screened for expression of bacHaPrP<sup>C</sup> by SDS-PAGE and Western immunoblotting using the anti-PrP monoclonal antibody 3F4 (Kascak et al., 1987). One stable, high expression virus (BacIII-HaPrP<sup>P</sup>) was selected and used for the remainder of the study.

**Purification of the 35S-bacHaPrP<sup>P</sup>.** Cells expressing bacHaPrP<sup>C</sup> were labelled for 3 h with 1 mCi [35S]methionine/I<sup>125</sup>S)cysteine (Expre<sup>S</sup>S/S Protein labelling mix, DuPont–NEN) per 25 cm<sup>2</sup> flask of 80% confluent cells in methionine- and cysteine-deficient Grace's insect medium (Sigma). The cells were lysed in ice with LB buffer (0.05 M Tris–HCl, pH 7.4; 0.15 M NaCl; 0.5% Triton X-100; 0.5% sodium deoxycholate) and the radiolabelled proteins were immunoprecipitated in buffer A (0.05 M Tris–HCl, pH 8.2; 0.15 M NaCl; 2% [w/v] N-laurylsarcosine; 0.4% [w/v] lecithin) using the 3F4 antibody and protein A-Sepharose beads (Caughey et al., 1995). The 35S-bacHaPrP<sup>C</sup> was eluted from the antibody–protein A–Sepharose complex in 0.1 M acetic acid and stored at 4 °C (Kocisko et al., 1996).

**Animal bioassay.** BacHaPrP<sup>C</sup> was tested for infectivity using an animal bioassay. Syrian Golden hamsters (n = 8) were intracerebrally inoculated with 200–250 ng of the recombinant protein in PBS (50 µl). Hamsters were monitored for clinical signs of TSE for 400 days.

**Isolation of PrP<sup>Sc</sup>.** PrP<sup>Sc</sup>-enriched fractions were prepared from the brains of Syrian Golden hamsters (infected with either the hyper or drowsy strain of hamster-adapted TME) as described (Caughey et al., 1995).

**Protein concentration assay.** Protein concentrations were measured with the Bio-Rad protein assay.

**Cell-free conversion reaction.** PrP<sup>Sc</sup>-enriched preparations (1–2 mg/ml) were partially denatured by incubation in 2.5 M guanidine hydrochloride (Gdn-HCl) for 7 h at 37 °C. Aliquots of denatured PrP<sup>Sc</sup> (2–3 µg) and the 35S-bacHaPrP<sup>P</sup> solution (50 ng) were mixed, diluted to 1 M Gdn-HCl in conversion buffer (1% N-laurylsarcosine; 5 mM cetylpyridinium chloride; 50 mM sodium citrate, pH 6.0), sonicated for 10 s and incubated at 37 °C for 2 days (Caughey et al., 1995). To measure proteinase K resistance to PK, the samples were treated with 25 µg/ml PK at 37 °C for 1 h. A PK inhibitor (Pefabloc; Boehringer Mannheim) and 20 µg of a carrier protein (thromboglobulin) were added and the proteins were precipitated in 4 vols of methanol at −20 °C. The resulting pellet was boiled in sample buffer and fractionated by SDS–PAGE. Conversion of 35S-bacHaPrP<sup>C</sup> was analysed by the presence and size of the protease-resistant 35S-labelled material (35S-bacHaPrP<sup>P</sup>-res) by autoradiography and quantified using a Phosphomager (Molecular Dynamics) and ImageQuant software.

**Results**

**Expression and characterization of HaPrP<sup>C</sup> in baculovirus-infected cells.** HaPrP<sup>C</sup> was synthesized in insect cells infected with recombinant virus BacIII-HaPrP<sup>C</sup>. Cell lysates were analysed by Western blot analysis using an anti-PrP monoclonal antibody, 3F4. The recombinant protein exhibited a molecular mass of 26–30 kDa (Fig. 1A). The protein was detected as early as 16 h post-infection (p.i.) (Fig. 1B) and was largely virion-associated at 3 days p.i. The yield (quantified by Western blot analysis) was approximately 5 mg of HaPrP<sup>C</sup> per litre of culture medium.

The baculovirus expression system produced a protease-sensitive PrP<sup>C</sup> (PrP-sen) that was completely degraded after PK treatment (25 µg/ml, 1 h, 37 °C; Fig. 1A). BacHaPrP<sup>C</sup> was not infectious as no clinical symptoms of scrapie were observed in hamsters inoculated intracerebrally with the recombinant protein (>1 year).

**Conversion of bacHaPrP<sup>C</sup> to protease-resistant form**

To determine whether bacHaPrP<sup>C</sup> could act as a substrate for conversion to a PK-resistant form in a cell-free system, 35S-
bacHaPrP<sup>C</sup> was incubated for 2 days with partially denatured PrP<sup>HY</sup>. PK-resistant <sup>35</sup>S-bacHaPrP<sup>C</sup> was observed after PK digestion. The PK digestion products exhibited a 6–7 kDa decrease in molecular mass as expected for PK cleavage of PrP<sup>C</sup> (Fig. 2A). PK-resistant <sup>35</sup>S-bacHaPrP<sup>C</sup> was incubated under the same conditions in the absence of PrP<sup>Sc</sup>-enriched preparations was completely digested by PK (Fig. 2A), indicating that the conversion was PrP<sup>Sc</sup>-dependent. The conversion efficiencies of <sup>35</sup>S-bacHaPrP<sup>C</sup> to <sup>35</sup>S-bacHaPrP-res were ~ 25–30%. <sup>35</sup>S -PrP-res products were not observed when more extensively denatured PrP<sup>Sc</sup> (6 M Gdn-HCl) was used in the reaction (data not shown). The conversion of bacHaPrP<sup>C</sup> into protease-resistant forms increased as a function of time (Fig. 2A, B). Serial dilution of the PrP<sup>Sc</sup>-enriched preparations resulted in the absence of conversion at PrP<sup>Sc</sup> concentrations below 100 µg/ml (Fig. 2C), suggesting that a critical PrP<sup>Sc</sup> concentration was required for conversion.

### Strain specificity of the conversion reaction

The hyper and drowsy strains of hamster-adapted TME produce strain-specific abnormal PrP that can be distinguished after PK digestion. PrP<sup>HY</sup>, upon PK digestion, is 1–2 kDa larger in size than similarly treated PrP<sup>DY</sup> (Fig. 3A) (Bessen & Marsh, 1992a, b, 1994; Caughey et al., 1998). To determine whether bacHaPrP<sup>C</sup> was converted in a similar manner, we performed experiments using abnormal PrP from each strain. PrP<sup>HY</sup>- and PrP<sup>DY</sup>-enriched preparations drove the conversion of <sup>35</sup>S-bacHaPrP<sup>C</sup> into products with the sizes predicted after PK digestion. The observed 1–2 kDa size difference in the converted products after protease digestion (Fig. 3B) was reproducible using four independent PrP<sup>HY</sup> and four independent PrP<sup>DY</sup>-enriched preparations.

Another distinguishing characteristic of PrP<sup>HY</sup> and PrP<sup>DY</sup> is the increased susceptibility of PrP<sup>DY</sup> to protease digestion (Bessen & Marsh, 1994). PK digestion (100 µg/ml, 37 °C) results in an 80% reduction of <i>in vivo</i> generated PrP<sup>DY</sup> after 1 h, whereas a similar reduction in PrP<sup>HY</sup> requires 48 h (Bessen & Marsh, 1994). To determine whether this characteristic was also transferred from PrP<sup>Sc</sup> to radiolabelled bacHaPrP<sup>C</sup>, a PK-digestion time-course (0–5–2 h, 50 µg/ml, 37 °C) and protease digestion under increasing concentrations of PK (25–100 µg/ml for 1 h at 37 °C) of the strain-specific converted products were performed. In both sets of experiments, the DY-derived <sup>35</sup>S-bacHaPrP-res product was less resistant to PK digestion than the HY-derived product (Fig. 4A, B). To further characterize the differential PK sensitivity, the effect of
Fig. 4. Propagation of two distinguishing strain-specific properties into $^{35}$S-bacHaPrP$^C$. The conversion products driven by PrP$^{HY}$ and PrP$^{DY}$ display differences in the PK-cleavage site as well as the relative resistance to PK. Molecular mass markers are indicated to the right. (A) Converted products driven by PrP$^{HY}$ and PrP$^{DY}$. PK-digestion time-course of the conversion products driven by equivalent amounts of PrP$^{HY}$ and PrP$^{DY}$. Conversion reactions were digested with PK (50 µg/ml) at 37 °C for increasing periods of time (30 min–2 h). The presence of minor converted products can be observed in both reactions. (B) Converted products driven by PrP$^{HY}$ and PrP$^{DY}$. Conversion reactions driven by equivalent amounts of PrP$^{HY}$ and PrP$^{DY}$ were digested with increasing concentrations of PK (25–100 µg/ml, 1 h, 37 °C). The presence of minor converted products can be observed in both reactions. (C) Percentage of remaining PK resistance of the converted products (data represent an average of three independent reactions ± standard deviations). Conversion reactions were divided into three equal aliquots and digested with 25, 50 or 100 µg/ml PK (1 h, 37 °C). The percentage of remaining PK resistance represents the percentage of $^{35}$S-bacHaPrP-res [% conversion = (vol. of protease-resistant radiolabelled material)/ (vol. of undigested PrP) x 100] obtained after PK digestions (at 50 and 100 µg/ml) normalized to the percentage of $^{35}$S-bacHaPrP-res present at 25 µg/ml. Shaded and black bars represent conversion products driven by PrP$^{HY}$ and PrP$^{DY}$, respectively.

increasing PK concentrations was measured between the strain conversion products. The percentage of PK-resistant material obtained after PK digestions (25, 50 and 100 µg/ml for 1 h at 37 °C) was quantified and normalized as a percentage to the amount present at 25 µg/ml. As shown in Fig. 4(C), the percentage of the DY-derived $^{35}$S-bacHaPrP-res product that remained resistant to 50 and 100 µg/ml PK was significantly lower ($P < 0.05$, Student’s t-test) than the HY-converted material under both conditions. These results suggest that the PK-resistant material generated by the in vitro conversion of $^{35}$S-bacHaPrP$^C$ into $^{35}$S-bacHaPrP$^{HY}$ and $^{35}$S-bacHaPrp$^{DY}$ exhibited two defining strain characteristics: (i) differential PK cleavage site and (ii) differing susceptibility to PK digestion.

Discussion

The purification difficulties and low yield of cellular PrP$^C$ obtained from brain tissue (Turk et al., 1988; Pergami et al., 1996) has led to the use of various recombinant expression systems including E. coli (Weiss et al., 1995; Mehlhorn et al.,...
in vitro demonstration that recombinant HaPrP is converted into the PK-resistant form upon interaction with PrPSc. We report here the expression of HaPrPSc in baculovirus-infected insect cells. The expressed protein, containing a processed full-length form of HaPrP (23–231), displayed predominantly a molecular mass of 27 kDa corresponding to the unglycosylated HaPrPSc form. The presence of partially glycosylated minor isoforms were also observed (28–30 kDa) but in much lower quantities. In contrast, HaPrPSc obtained from uninfected brain tissues has a molecular mass of 33–35 kDa (Oesch et al., 1985), as a result of GPI anchor addition and glycosylation. These findings suggest that insect cell lines do not generate the complex post-translational modifications present in mammalian cells. Similar results were obtained using a different baculovirus expression vector (Scott et al., 1988).

The ‘prion hypothesis’ predicts that PrPSc display self-converting activity by interacting with PrPSc (Prusiner, 1998). We tested the ability of bacHaPrP to acquire a PrPSc-like conformation in the presence of PrPSc-enriched preparations. Since previous cell-free conversion experiments used recombinant PrPC expressed in mammalian cell lines or PrP immunoprecipitated from brain homogenates, it is possible that other putative factor(s) may copurify with the prion protein and affect these reactions (Westaway et al., 1998). A number of studies have shown that bacterial chaperones (DeB Burman et al., 1997), transition metals (McKenzie et al., 1998, 1999) and cell lysates (Saborio et al., 1999) can facilitate the in vitro formation of PK-resistant protein. Our data demonstrate that recombinant HaPrPSc synthesized in a non-mammalian expression system can be converted in vitro into PK-resistant form upon interaction with PrPSc-enriched preparations. Since 35S-bacHaPrPSc and PrPSc molecules constituted the main components of the assay, our data indicate that the formation of PrP-res is the result of the PrPSc self ability to propagate. These results also reinforce the specificity of the PrPSc–PrPSc interactions and the utility of the cell-free assay, although they do not eliminate the possible requirement of accessory molecules that may be essential and/or influence PrP-res formation. Similar to conversion studies using mammalian HaPrPSc (Kocisko et al., 1994; Bessen et al., 1995), post-translational modifications of PrPSc do not seem to be required for the conversion reaction. This suggests that, independent of the cell source where the primary polypeptide was expressed, the conformation(s) imposed by the primary HaPrP sequence is sufficient for its ability to act as a substrate in the in vitro assay. Correspondingly, the converting activity of PrPSc was also functional upon interacting with bacHaPrPSc. Our data, therefore, represent the first study of functional PrPSc-converting activity of non-mammalian derived HaPrPSc.

The acquisition of PK resistance of baculovirus-expressed HaPrPSc upon incubation with partially denatured PrPSc, including the specific PK-cleavage site characteristic of PrPSc, is time-dependent and requires a critical PrPSc concentration. This pattern was consistent with other studies (Caughey et al., 1995, 1997), indicating that PrPSc aggregates are critical for converting activity and support the nucleated polymerization model for the PrPSc-to-PrPSc transition (Harper & Lansbury, 1997). The slight size increase of the converted material observed during the time-course of 35S-bacHaPrPSc conversion (Fig. 2A) suggests that, in the early stages of the reaction, the converted material adopts a conformation that is less structured and, therefore, more accessible to PK digestion. Efficiency levels for the formation of 35S-bacHaPrPSc-res (25–30%) are comparable to cell-free conversions using mammalian cell-derived recombinant PrPSc (Caughey et al., 1995; Raymond et al., 1997; Bossers et al., 1997). In some conversion reactions, two different bands were observed (Fig. 2C), possibly a result of gel running conditions.

To further characterize the specificity of the cell-free conversion assay using baculovirus-expressed PrPSc, PrPPhy- and PrP DY-enriched preparations were tested for their ability to convert bacHaPrPSc into strain-specific products. We characterized the conversion through the analysis of two strain-specific biochemical markers, the molecular size difference after PK digestion and the degree of PK resistance. As a result of conversion of recombinant PrPSc driven by either PrPHy or PrP DY, the converted material displays not only the electrophoretic mobility shift characteristic for each strain after PK digestion (Bessen et al., 1995) but also the relative resistance to PK. These data indicate that, upon propagation of the PrPSc state, insect cell-derived HaPrP can acquire different PrPSc conformations and that strain-specific PrP-res properties can be reproduced when this source of PrP was used as a conversion substrate. The acquisition of both strain biochemical markers, the molecular size difference after PK digestion (Harper & Lansbury, 1998), and at the molecular level, strain properties of hamster-adapted TME agent.

In hamster-adapted TME, PrP DY accumulates at a slower rate than PrPHy (McKenzie et al., 1996). These observations could result from strain-specific differences in conformational conversion rates and/or metabolic clearance of the converted products. The efficiency of conversion using our standard PK digestion treatment (25 µg/ml, 1 h, 37 °C) was 25–30% (±7%) in both PrPHy- and PrP DY-derived products. Under these conditions, we do not observe differences in conversion activity between the two strains. There is, however, a remarkable effect on the strain-properties of the converted products when higher concentrations of PK or longer protease-digestion periods are used (Fig. 4). As time of digestion and/or PK concentration increase (50–100 µg/ml), the 35S-DY-like converted product exhibits a higher susceptibility to PK (Fig. 4).
 Altogether these data support the self-propagating activity of differential turnover of PrP\(^{HY}\) and PrP\(^{DY}\) could account for the differences observed in the incubation period upon their transmission into hamsters. In contrast to these observations, Bessen et al. (1995) reported different converting efficiencies between the strain-specific converted products. Furthermore, the investigators did not detect differences in PK sensitivity in the converted products. The discrepancies with our observations are perhaps the result of different experimental conditions used in the assay (conversion buffer composition, recombinant HaPrP source, PK concentrations and digestion times). It should be emphasized that, similar to our findings, in vivo PrP\(^{HY}\) is more resistant to PK than PrP\(^{DY}\).

Although the infectious nature of the converted products remains to be determined, we have demonstrated that recombinant HaPrP expressed in non-mammalian systems can be converted in vitro into PrP-res in the presence of PrP\(^{Sc}\). Moreover, conformational differences between PrP\(^{HY}\) and PrP\(^{DY}\) that account for their strain-specific biochemical properties were faithfully transferred into insect cell-derived HaPrP. Altogether these data support the self-propagating activity of PrP\(^{Sc}\). It has been proposed that the in vitro PrP conformational transition requires the presence of molecular chaperones (Prusiner, 1998). The use of defined components in the conversion reactions should facilitate the identification of potential cofactors involved in PrP\(^{Sc}\) formation.

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