The transmissible spongiform encephalopathies (TSEs) comprise a group of fatal neurodegenerative diseases that are characterized by the conversion of the normal host cellular prion protein (PrP\textsuperscript{C}), to the abnormal protease-resistant prion protein isoform (PrP\textsubscript{res}). It has been proposed, though not proven, that the infectious TSE agent consists solely of PrP\textsubscript{res} and that PrP\textsubscript{res}-induced conformational conversion of PrP\textsuperscript{C} to additional PrP\textsubscript{res} represents agent replication. In this study we demonstrate in situ conversion of protease-sensitive PrP\textsuperscript{C} to PrP\textsubscript{res} in TSE-infected brain slices. One step in this process is the binding of soluble PrP\textsuperscript{C} to endogenous PrP\textsubscript{res} deposits. The newly formed PrP\textsubscript{res} is associated with the slices in a pattern that correlated with the pre-existing brain distribution of PrP\textsubscript{res}. Punctate in situ PrP conversion was observed in brain regions containing PrP\textsubscript{res} amyloid plaques, and a more dispersed conversion product was detected in areas containing diffuse PrP\textsubscript{res} deposits. These studies provide direct evidence that PrP\textsubscript{res} formation involves the incorporation of soluble PrP\textsuperscript{C} into both nonfibrillar and fibrillar PrP\textsubscript{res} deposits in TSE-infected brain. Our findings suggest that the in situ PrP conversion reaction leads to additional polymerization of endogenous PrP\textsubscript{res} aggregates and is analogous to the process of PrP\textsubscript{res} fibril and subfibril growth in vivo.

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**In Situ** Formation of Protease-resistant Prion Protein in Transmissible Spongiform Encephalopathy-infected Brain Slices* (Received for publication, March 20, 1997, and in revised form, April 7, 1997)

The transmissible spongiform encephalopathies (TSEs), or prion diseases, comprise a group of fatal neurodegenerative diseases of humans and animals. The past decade has witnessed the emergence of two new TSEs, bovine spongiform encephalopathy (1) and a variant form of human Creutzfeldt-Jakob disease (2), and the discovery of another human TSE, fatal familial insomnia (3, 4). In yeast, two phenotypes, [URE3] and [PSI+] have been attributed to non-Mendelian genetic elements that have similarities to both infectious and genetic properties of the TSEs (5, 6). However, little is known about the molecular details concerning the propagation of the TSE agents and related yeast phenotypes.

The unusual nature of the agent causing the TSEs has led to the proposal that these agents are devoid of a nucleic acid genome and consist solely of protein (7, 8). The discovery of scrapie-associated fibrils in the early 1980s (9) paved the way to the identification of a protease-resistant protein (8, 10) that was a major component of scrapie-associated fibrils (10) and unique to TSE-infected hosts. This protein, called the scrapie prion protein (PrP\textsuperscript{Sc}) or protease-resistant PrP (PrP\textsubscript{res}) (8), appears to be a conformational isomer of a cellular homologue called PrP\textsuperscript{C} (11). Although PrP\textsuperscript{C} is soluble, monomeric, and readily degraded by proteases, PrP\textsubscript{res} has a high β-sheet content characteristic of an amyloidogenic protein polymer (12), assembles into fibrils both in vivo (13) and in vitro (10, 14–16), and is a component of amyloid plaques (17, 18). The TSEs are similar to other amyloidogenic diseases in that they can have either a sporadic etiology or be inherited diseases that are linked to mutations in the open reading frame of the host amyloid precursor gene, in this case the prion protein gene. However, the TSEs are also infectious diseases, and there is strong experimental data suggesting that PrP\textsubscript{res} is the main component of the TSE agent, although this has not been conclusively demonstrated.

The mechanism of PrP\textsubscript{res} formation has been investigated in a cell-free conversion reaction in which \[^{35}S\]methionine-labeled PrP\textsuperscript{C} (\[^{35}S\]PrP\textsuperscript{C}) is converted into proteinase K (PK)-resistant \[^{35}S\]-PrP (\[^{35}S\]-PrP\textsubscript{res}) in the presence of TSE brain-derived PrP\textsubscript{res} (19). Both the brain-derived PrP\textsubscript{res} and nascent \[^{35}S\]-PrP\textsubscript{res} are reduced in molecular mass by 6–7 kDa after PK digestion, whereas any unconverted \[^{35}S\]-PrP\textsuperscript{C} is completely degraded. The formation of \[^{35}S\]-PrP\textsubscript{res} cell-free conversion products requires the presence of PrP\textsubscript{res} aggregates (20). Irreversible denaturation of PrP\textsubscript{res} structure results in the coincident loss of conversion activity, resistance to PK, and a decrease in TSE infectivity (21). Furthermore, the cell-free conversion reaction exhibits both TSE strain and species specificity (22–24), which suggests it is analogous to biological processes that occur in vivo. In this study we investigated PrP\textsubscript{res} formation in TSE-infected brain tissue by using intact brain slices instead of purified PrP\textsubscript{res} in the cell-free conversion reaction. This in situ PrP conversion assay provides a powerful tool to model PrP\textsubscript{res} formation in vivo.

**EXPERIMENTAL PROCEDURES**

**Source of TSE Agent**—Brain tissue was collected from clinically ill Syrian golden hamsters (outbred LVK/LAK) inoculated with the HY strain of TME as described previously (25).

**Cell-free Conversion Reaction**—The cell-free conversion reaction was performed by incubating ~1.6 µg of brain-derived PrP\textsubscript{res} (prepared as described previously (26) except the proteinase K digestion step was omitted) with \[^{35}S\]-PrP\textsuperscript{C} (20,000 cpm, ~3 ng) lacking its glycosphatidylinositol C-terminal anchor that was purified from murine fibroblast cells transfected with the Syrian hamster PrP gene as described previ-
RESULTS

To investigate if the PrP conversion reaction could occur in vivo, we tested whether brain slices from hamsters infected with the HY strain of TME (25, 26), a TSE of mink, could induce PrP conversion. Fig. 1 illustrates that a brain slice from a TME-infected hamster, but not from an uninfected host, can induce conversion of labeled PrP in the presence of brain-derived PrP-res. The conversion reaction solution incubating on top of the TME-infected brain slice contained labeled PrP that was degraded by PK and did not contain PrP-res (data not shown). These results indicate that TSE infected slices can induce PrP conversion and that one step in the conversion is the binding of labeled PrP to PrP-res. Furthermore, a portion of the labeled PrP was converted into a PK-resistant and insoluble PrP molecule similar to the original PrP-res aggregate. These results suggest that these properties of the conversion products are due to the incorporation of labeled PrP into the pre-existing PrP-res polymer, but not all aggregation or binding of labeled PrP to PrP-res results in conversion to PrP-res.

Based on these observations, we developed an in situ PrP conversion assay to determine the spatial localization of converting activity in intact TME-infected brain slices. The conversion reaction was performed as described in Fig. 1, except the brain slices were left on the slide and analyzed by film and liquid emulsion autoradiography. Fig. 3B illustrates conversion of labeled PrP to PrP-res in specific anatomical structures in the TME-infected hamster brain but not in the uninfected brain (Fig. 3A). Brain regions containing the most intense PrP conversion include the thalamus, hippocampus, and the middle cerebral cortical layers (Fig. 3B). PrP-res Conversion in Brain Slices

Fig. 1. In situ cell-free conversion reactions were performed by incubating labeled PrP with unfixed brain slices attached to microscope slides from uninfected or TME-infected hamster brain prior to and after conversion. Immediately after the cell-free conversion reaction was initiated (t₀), the total (T) reaction mixture was separated into pellet (P) and supernatant (S) fractions by microcentrifugation. This separation procedure was repeated on a parallel reaction after the conversion reaction was carried out for 24 h (t₁). The pellet and supernatant fractions were analyzed with and without PK treatment (middle and top panels, respectively) followed by SDS-PAGE and fluorography to detect labeled PrP. Total nonradioactive PrP-res was analyzed after PK digestion by Western blotting (bottom panel). The ratio of reaction equivalents represented in the lanes of the top (−PK) and middle (+PK) panels is 1:8.
that contained PrP-res deposits but also for the hamster 35S-PrPC precursor because no in situ PrP conversion was observed after incubation with murine 35S-PrPC (Fig. 3C). The absence of PrP conversion in the latter case is consistent with the inability of hamster-adapted scrapie to cause experimental disease in mice (29, 30) and also the inability of purified hamster PrP-res to convert murine 35S-PrPC to a PK-resistant form in the cell-free conversion reaction (23). The in situ conversion assay, in which the brain cytoarchitecture is maintained, demonstrates that PrP conversion occurs in specific anatomical structures just as PrP-res deposits are distributed in a distinct pattern in vivo (31).

When the distribution of endogenous PrP-res was compared with in situ PrP conversion activity, an excellent correlation was found. Fig. 4A illustrates a similar distribution of endogenous PrP-res, detected by immunocytochemistry, and 35S-PrPC conversion activity in the thalamus, hippocampus, and middle cerebral cortical layers. In brain regions that lack PrP-res (e.g., corpus callosum and the molecular layer of the dentate gyrus), no conversion activity was observed, indicating that in situ formation of 35S-PrPC-res was PrP-res-dependent and did not redistribute to areas lacking PrP-res deposits. In the reticular thalamic nucleus and ventral posterolateral thalamic nucleus the co-distribution of endogenous PrP-res and PrP conversion activity appeared to be confined to the gray matter and not the white matter tracts traversing these nuclei (Fig. 4B). This co-distribution pattern also appeared to be quantitative because the intensity of immunostaining for pre-existing PrP-res correlated with the relative intensity of in situ conversion activity. In Fig. 4B, the reticular thalamic nucleus contained the highest levels of PrP-converting activity and immunostaining, whereas the internal capsule was limited to a sparse, uncincentric distribution. These data indicate that the distribution and amount of PrP-res deposits, and not PrP-independent host factors, were the major determinants for the location and intensity of in situ PrP conversion activity.

At the microscopic level, there was also a good correlation between the nature of the PrP-res deposit and the 35S-PrP conversion product. PrP-res accumulates into amyloid plaques in specific brain regions in TSE-infected hamsters. One prominent region is the subependymal cell layer adjacent to the lateral ventricles (17). When conversion activity was detected by liquid emulsion autoradiography, the converted 35S-PrP-res products had a punctate pattern in this location (Fig. 4C),
suggesting that \textit{in situ} growth of PrP-res plaques had occurred. In brain areas with a diffuse pattern of PrP-res deposition in which there is no defined PrP ultrastructure (32, 33), the $^{35}$S-PrP-res conversion products had a more dispersed pattern (Fig. 4D). These patterns of \textit{in situ} conversion precisely mimic endogenous PrP-res deposits regardless of whether the PrP-res had a distinguishable ultrastructure and provide evidence for the incorporation of $^{35}$S-PrP directly into endogenous PrP-res.

**DISCUSSION**

We have used the cell-free and \textit{in situ} PrP conversion reactions to understand the mechanism involved in the conversion of $^{35}$S-PrP$^{C}$ into $^{35}$S-PrP-res. These \textit{in vitro} models mimic important biochemical and biological aspects of TSE diseases. For one, the cell-free conversion PrP products, like brain-derived PrP-res, are insoluble and truncated by 6–7 kDa compared with the soluble PrP$^{C}$ precursor (Ref. 19 and this study); this provides evidence that the \textit{in vitro} and \textit{in vivo} derived PrP-res aggregates have the same conformation. Both the cell-free and \textit{in situ} PrP conversion reactions exhibit species specificity that is similar to interspecies transmission of scrapie between hamster and mouse (Ref. 23 and this study). Hamster-adapted scrapie agent (263K) does not induce experimental TSE disease in mice (29, 30), and this could be explained at the molecular level by the failure of hamster PrP-res to convert murine $^{35}$S-PrP$^{C}$ to a protease-resistant form in both the cell-free (23) and \textit{in situ} PrP conversion reactions. Additional experiments have demonstrated that the susceptibility of sheep to natural scrapie, which is largely influenced by PrP gene polymorphisms, correlates with the effect of PrP$^{P}$ sequence variability in the cell-free conversion reaction (24). $^{35}$S-PrP$^{C}$ derived from two PrP genotypes associated with high susceptibility to scrapie were converted to $^{35}$S-PrP-res in the cell-free conversion reaction with much greater efficiency than PrP$^{C}$ from a scrapie-resistant PrP genotype, indicating that this \textit{in vitro} assay closely parallels important aspects of TSE pathogenesis. Another biological characteristic of TSE diseases that has been modelled using the cell-free conversion reaction is TSE strain diversity (22). The protein-only model predicts that inheritance of TSE strain phenotypes is mediated by stable propagation of different PrP-res conformations. The HY and DY TME strains have different size PrP-res fragments and additional strain-specific biochemical properties that indicate the PrP-res from the two strains have distinct three-dimensional conformations (26). When HY and DY PrP-res are used in the cell-free conversion reaction, they can convert the same $^{35}$S-PrP$^{C}$ into differently structured PrP-res molecules, indicating that this reaction leads to conformation-specific PrP conversion that is determined by the strain of PrP-res (22). These studies strongly suggest that the molecular basis of TSE strain diversity lies within the conformation of PrP-res. The distinct conformers of PrP-res can self-propagate by a nongenetic mechanism and, because they are linked to the infectious TME agent (26), may themselves induce different clinicopathological diseases. These studies demonstrate that the cell-free and \textit{in situ} PrP conversion reactions are useful models for reconstructing both biochemical and biological aspects of TSE diseases.

The \textit{in situ} PrP conversion assay uses intact brain slices to maintain tissue cytoarchitecture and therefore likely resembles the \textit{in vivo} process of PrP-res formation. Unlike our previous studies where the purified PrP-res was typically partially unfolded in guanidine hydrochloride prior to the cell-free conversion reaction (19–23, 34), PrP-res was not denatured in the \textit{in situ} PrP conversion, suggesting that native PrP-res structure is capable of inducing conversion \textit{in vivo}. \textit{In situ} PrP conversion was PrP-res-dependent, involved binding of $^{35}$S-PrP$^{C}$ to PrP-res, and resulted in the conversion of soluble, PK-sensitive $^{35}$S-PrP$^{C}$ into an insoluble and PK-resistant PrP molecule. Furthermore, this study directly demonstrated \textit{in situ} formation of $^{35}$S-PrP-res at the sites of endogenous PrP-res deposits. The $^{35}$S-PrP conversion products were associated with both PrP amyloid plaques, which often consist of radial arrays of PrP-res fibrils and diffuse PrP-res deposits which can consist of individual PrP-res fibrils or have no distinguishable ultrastructural morphology (32, 33). Thus, it is likely that the mechanism for \textit{in situ} PrP conversion is the same for both types of PrP-res deposits. Our findings strongly suggest that the soluble PrP$^{C}$ precursor is incorporated as a subunit of the PrP-res deposit and results in additional polymerization of the pre-existing PrP-res aggregate. A similar finding has been demonstrated upon incubation of synthetic $\beta$-amylloid peptide with brain slices from Alzheimer’s disease (35, 36).

Several models for PrP-res formation have been proposed, including chaperone-mediated mechanisms (37–39), nucleation-dependent polymerization (7, 40–43), and a heterodimer interaction (7, 44). The cell-free conversion reaction provides valuable information that can be used to evaluate these models. Based on our studies we think it is unlikely that chaperones are required in the conversion of PrP$^{P}$ to PrP-res because only purified PrP molecules are needed in the cell-free conversion reaction. Treatment of PrP-res with PK prior to the cell-free conversion reaction eliminates most if not all proteins copurifying with PrP-res but does not disrupt conversion activity (20, 22), suggesting that potential cofactors must also be PK-resistant. However, in the case of yeast prions, the formation of the TSE-like [PSI$^{+}$] phenotype is dependent on the functional state of the heat shock protein (Hsp)104 chaperone (38, 39) and led to the proposal that Hsp104 acts directly at the step(s) involved in the conversion from the normal [psi$^{-}$] to the [PSI$^{+}$] phenotype by partially unfolding the sup35 protein before it aggregates to the [PSI$^{+}$] phenotype (38, 39). In our cell-free conversion reactions $^{35}$S-PrP$^{C}$ is likely denatured by acetic acid during purification but presumably refolds during the conversion reaction. Therefore, prior unfolding of PrP$^{C}$ may mimic the action of Hsp104-like chaperones, raising the possibility that \textit{in vivo} conversion to PrP-res requires chaperone-assisted unfolding of PrP$^{C}$. Alternatively, chaperones may mediate proper folding of PrP$^{C}$ (45), which may be prerequisite for conversion to PrP-res.

The cell-free conversion reaction studies indicate that PrP conversion activity is associated with a PrP-res aggregate; monomers or small oligomers of PrP derived from a PrP-res aggregate do not convert $^{35}$S-PrP$^{C}$ to PrP-res (20). PrP-res aggregates containing PrP converting activity are associated with high levels of TSE infectivity. In concentrations of guanidine hydrochloride that result in a reduction of PrP conversion activity there is a coincident loss of TSE infectivity (21). These findings and our present observations that $^{35}$S-PrP becomes bound to endogenous PrP-res deposits during \textit{in situ} PrP conversion are consistent with PrP-res formation based on nucleation-dependent protein polymerization. This is a two-step process that involves nucleus formation and polymerization or growth of the aggregate (41, 42, 46). Nucleus formation is rate-limiting under normal physiological conditions due to the kinetic unlikelihood for PrP$^{C}$ monomers to randomly associate into a small, stable multimers. However, in the presence of a PrP-res aggregate, as in the case of experimental TSE inoculation, the addition of monomeric PrP$^{P}$ to the PrP-res nucleus is stabilized by intermolecular interactions between PrP$^{P}$ and multiple PrP-res molecules at the nucleation site. This can lead to a conformational change of PrP$^{P}$ to PrP-res, growth of the PrP-res polymer and, over time, amylloid fibril formation. In our cell-free conversion reaction we are likely providing the PrP-res aggregate or nucleus that can seed the growth of the
PrP polymer by the addition of monomeric PrPC. This model predicts that the location of newly formed \( ^{35}S\)-PrP should be at the site of pre-existing PrP-res. In our \textit{in situ} PrP conversion reaction, the \( ^{35}S\)-PrP remained bound to the brain slice in a distribution analogous to that found for endogenous PrP-res. This is consistent with temporal studies in TSE-infected hosts where following initial PrP-res deposition there is additional accumulation of PrP-res in these brain regions (31, 47).

Based on these results we find no evidence to support the heterodimer model (7, 44) for PrP-res assembly. This model predicts that a single PrP-res molecule forms a dimer with monomeric PrPC and induces a conformational change to produce a PrP-res homodimer that then dissociates. In the heterodimer model PrP-res conversion activity would be associated with monomeric PrP-res but in the \textit{in situ} PrP conversion reaction we do not detect soluble \( ^{35}S\)-PrP-res. The newly formed \( ^{35}S\)-PrP co-sediments with the brain-derived PrP-res after centrifugation at 12,000 \( \times \) g for 20 min, and the soluble \( ^{35}S\)-PrP remaining in the supernatant fraction was PK-sensitive. Our conclusions are supported by the findings that both PrP-res and TSE infectivity are tightly associated with membrane fractions from TSE-infected brain extracts and not with soluble tissue extracts (48). Furthermore, purified PrP-res is extensively aggregated, insoluble in non-denaturing detergents, and associated with high levels of TSE infectivity (10). These data suggest that if the TSE agent is demonstrated to consist solely of PrP-res, then a PrP-res aggregate or nucleus, but not a monomer, is the infectious form. It is possible that a putative monomeric PrP-res molecule could bind to other macromolecules, such as proteoglycans (49, 50), that may copurify with PrP-res. This could give it the size characteristics of a PrP-res multimer, however, PK treatment of PrP-res preparations prior to the cell-free conversion reaction does not dissociate converting activity from the PrP-res aggregate, so such potential cofactors would have to be PK-resistant (20). Furthermore, there is no evidence to suggest that the self-assembly of amyloidogenic proteins in general proceeds via the heterodimer mechanism, but there is support for nucleation-dependent polymerization (35, 36, 40–42).

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