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Nonenzymatic Glycation at the N Terminus of Pathogenic Prion Protein in Transmissible Spongiform Encephalopathies*

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Transmissible spongiform encephalopathies (TSEs) are transmissible neurodegenerative diseases characterized by the accumulation of an abnormally folded prion protein, termed PrP^{Sc}, and the development of pathological features of astrogliosis, vacuolation, neuronal cell loss, and in some cases amyloid plaques. Although considerable structural characterization of prion protein has been reported, neither the method of conversion of cellular prion protein, PrP^{C}, into the pathogenic isoform nor the post-translational modification processes involved is known. We report that in animal and human TSEs, one or more lysines at residues 23, 24, and 27 of PrP^{Sc} are covalently modified with advanced glycosylation end products (AGEs), which may be carboxymethyl-lysine (CML), one of the structural varieties of AGEs. The arginine residue at position 37 may also be modified with AGE, but not the arginine residue at position 25. This result suggests that nonenzymatic glycation is one of the post-translational modifications of PrP^{Sc}. Furthermore, immunostaining studies indicate that, at least in clinically affected hamsters, astrocytes are the first site of this glycation process.

PrP^{Sc},[1] which is the pathogenic isoform of normal cellular prion protein, termed PrP^{C}, is a biochemical marker for the diagnosis of human and animal TSEs. TSEs include bovine spongiform encephalopathy (BSE) in cattle, variant Creutzfeldt-Jakob Disease (vCJD) in humans and scrapie in sheep. Diagnosis of TSEs can be accomplished by a variety of tests using monoclonal and polyclonal antibodies, which identify PrP^{Sc}. Several recent studies report additional techniques for the detection of PrP^{Sc} involving repeated sonication of PrP^{C} in the presence of PrP^{Sc} seed, protein misfolding cyclic amplification (PMCA) (1), and the identification of $uPrP^{Sc}$ in the urine of TSE-positive animals and humans (2). Early diagnosis, therapy and prophylaxis of the TSEs are considered to be very important problems that confront the medical community. Solutions to these problems will be advanced by understanding the details of the conversion of PrP^{C} to PrP^{Sc} and the mechanisms involved in PrP^{Sc} accumulation. However, the post-translational modifications involved in the conversion of the host-coded glycoprotein into its pathogenic isoform are poorly understood.

Nonenzymatic glycation between reducing sugars and amino groups of proteins, termed the Maillard reaction, produces reversible Schiff bases and Amadori products. These early glycated products undergo further complex and advanced glycation and oxidation (glycoxidation), which elicits irreversible modification, to form heteromorphic and fluorescent derivatives termed AGEs (3, 4). AGEs can be synthesized *in vitro* by glycoxidation between reducing sugars, such as glucose (although compounds including glycolaldehyde, glyoxal, and methylglyoxal are more reactive than glucose *in vitro*), and proteins such as bovine serum albumin (BSA), RNase, and collagen (5, 6). Amino groups that are located in the side chains of amino acids, such as lysine and arginine, are primary targets for nonenzymatic glycoxidation with carbohydrates.

AGEs have been reported to be associated with pathogenesis in vascular disease, diabetes, atherosclerosis, renal failure, Alzheimer's disease (AD), and Parkinson's disease (PD), suggesting that AGEs may contribute to the progressive deterioration associated with these chronic diseases. Furthermore, the deleterious effects of AGEs may be associated with oxidative stress (7–12). It has been shown that tau, a protein associated with paired helical filaments (PHFs), which plays an important role in AD pathology, is advanced-glycated; AGE-positive immunostaining is present in the neuritic plaques and the neurofibrillary tangles (NFT) in AD brains (13). In addition, β_2-microglobulin, which is accumulated in amyloidosis and recombinant human interferon γ produced in *Escherichia coli*, are nonenzymatically glycated (14–16). The formation of AGEs is irreversible and causes protease-resistant modification of these peptides and proteins, leading to their deposition and to amyloidosis (17–19). These reports suggested an examination of the possibility that post-translational AGE-mediated modification of protease-resistant pathogenic PrP^{Sc} might play a role in protein stabilization and thereby be responsible, in part, for the accumulation of the aberrant protein in the brain.

Here we report that in samples taken just prior to the terminal stage of disease one or more lysine residues at position 23, 24, and 27 of PrP^{Sc} are covalently modified with AGEs,

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[1] The abbreviations used are: PrP^{Sc}, abnormally folded prion protein; TSEs, transmissible spongiform encephalopathies; PrP^{C}, cellular prion protein; AGEs, advanced glycosylation end products; CJD, Creutzfeldt-Jakob Disease; vCJD, variant Creutzfeldt-Jakob Disease; BSE, bovine spongiform encephalopathy; PK, proteinase K; BSA, bovine serum albumin; AGE-BSA, AGE-modified BSA; CML, carboxymethyl-lysine; AGE-HSA, AGE-modified human serum albumin; GFAP, glial fibrillary acidic protein; PNGase F, peptide *N*-glycosidase F; ELISA, enzyme-linked immunosorbent assay; d.p.i., days post-inoculation; TBS, Tris-buffered saline.

TABLE I
Sequences of synthesized prion peptides

Prion peptides composed of 14 residues were synthesized by peptide synthesizer (Model 431A) and purified by fast protein liquid chromatography (FPLC) (ÄKTA purifier 10/100, Amersham Biosciences) equipped with DELTA PAK C18 (3.9 × 150 mm, 5 μ, 300 Å, Waters Co.), and then lyophilized. Synthesized peptides were incubated with glucose for 90 days.

Seq. no.[a]	23		25					30					35					40					45					50
1	K	K	R	P	K	P	G	G	W	N	T	G	G	S														
2		K	R	P	K	P	G	G	W	N	T	G	G	S	R													
3			R	P	K	P	G	G	W	N	T	G	G	S	R	Y												
4				P	K	P	G	G	W	N	T	G	G	S	R	Y	P											
5					K	P	G	G	W	N	T	G	G	S	R	Y	P	G										
6						P	G	G	W	N	T	G	G	S	R	Y	P	G	Q									
7							G	G	W	N	T	G	G	S	R	Y	P	G	Q	G								
8								G	W	N	T	G	G	S	R	Y	P	G	Q	G	S							
9									W	N	T	G	G	S	R	Y	P	G	Q	G	S	P						
10										N	T	G	G	S	R	Y	P	G	Q	G	S	P	G					
11											T	G	G	S	R	Y	P	G	Q	G	S	P	G	G				
12												G	G	S	R	Y	P	G	Q	G	S	P	G	G	N			
13													G	S	R	Y	P	G	Q	G	S	P	G	G	N	R		
14														S	R	Y	P	G	Q	G	S	P	G	G	N	R	Y	
15															R	Y	P	G	Q	G	S	P	G	G	N	R	Y	P

[a] Number of peptides.

which may be carboxymethyl-lysine (CML), one of the structural varieties of AGEs. The arginine at position 37 may also be modified with AGE, but not the arginine residue at position 25. These results indicate that nonenzymatic glycation plays a role in the post-translational processing of PrP^{Sc}.

EXPERIMENTAL PROCEDURES

Animals and Scrapie Strains—Inbred mice (C57BL/6J, MB) and golden Syrian hamsters (SHa), 6 weeks of age, were obtained from the Experimental Animal Center of Hallym University, and divided into age-matched controls and groups destined to be infected with specific scrapie strains. Dr. Alan Dickinson of the Neuropathogenesis Unit (Edinburgh, Scotland) kindly provided the following scrapie strains: ME7 and 22L, which were passaged in C57BL mice, and 87V, which was passaged in MB mice. The 139A strain obtained from Dr. Richard H. Kimberlin of the Neuropathogenesis Unit (Edinburgh, Scotland) was passaged in C57 BL mice, and the 263K and 139H scrapie strains were passaged in hamsters. All passages were performed by intracerebral inoculation with 30 μl per mouse and 50 μl per hamster of a 1% (w/v) brain homogenate (prepared in 0.01 M phosphate-buffered saline (pH 7.4) from either normal brain or from scrapie-infected brain at the terminal stages of the disease). With the exception of the time course study (Fig. 3), 263K scrapie-infected hamsters were harvested at 70 days post-inoculation (d.p.i.), 22L scrapie-infected mice at 152 d.p.i., ME7, 139A, and 139H scrapie-infected animals at 158 d.p.i., and 87V scrapie-infected mice at 287 d.p.i. Animals were sacrificed when clinical signs of the disease were manifested. Animals were anesthetized with 16.5% urethane and then transcardially perfused with cold phosphate-buffered saline followed by cold 4% paraformaldehyde in phosphate-buffered saline for brain sections, whereas for the isolation of PrP^{Sc}-enriched insoluble fraction, each scrapie brain and age-matched control were frozen without perfusion and stored in −70 °C prior to use. Also, frozen portions of the temporal cortex of normal human and CJD brains and the frontal cortex of vCJD brain were used for isolation of PrP^{Sc}-enriched fractions.

Preparation of in Vitro AGE Products and Production of Anti-AGE Antibody—AGE-modified BSA (AGE-BSA), lysine-derived AGE (AGE-Lys), arginine-derived AGE (AGE-Arg), and the AGE-modification of PrP peptides (Table I) were prepared as previously described (20). Briefly, BSA, lysine, arginine (Sigma), and PrP peptides were dissolved with D-glucose (Sigma) in 0.5 M sodium phosphate buffer (pH 7.4). The solution was deoxygenated with nitrogen gas, and sterilized by ultrafiltration (0.45-micron filter, Nalgene), and then incubated at 37 °C for 7, 50, or 90 days, as noted in results. After incubation, the samples except for lysine- and arginine-derived AGE were dialyzed using three changes of 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The dialyzed samples and the D-glucose-treated lysine and arginine samples were then lyophilized. Rabbits were immunized with AGE-BSA as reported, and R3 anti-AGE antiserum was subsequently collected and purified with affi-gel protein A-agarose (Bio-Rad). Affinity-purified IgG was passed over a column of activated Sepharose 4B (Amersham Biosciences) coupled with BSA two times. The non-adsorbed fractions were combined and then passed over a column of AGE-BSA-coupled Sepharose 4B using a modification of a previous report (20). Anti-AGE IgG bound to AGE-BSA, which comes from what did not bind to BSA, was eluted with 0.1 M citric acid (pH 3.0) and then neutralized with 1.0 M Tris-HCl (pH 9.0). R3 anti-AGE antiserum and anti-AGE IgG purified from antiserum were used in the current study.

Isolation of PrP^{Sc}-enriched Insoluble Fraction—PrP^{Sc}-enriched insoluble fraction was isolated as previously described (21). Briefly, each normal and TSE brain was homogenized in Tris-buffered saline (TBS, pH 7.4), and then rehomogenized in TBS (pH 7.4) containing 20% *N*-lauroyl sarcosine (Sigma) and then centrifuged. The supernatant was ultracentrifuged and the resulting pellet resuspended in TBS (pH 7.4) containing 10% NaCl and 0.1% myristyl sulfobetaine (SB3-14) prior to treatment with or without proteinase K (PK) (Sigma). After the sample was ultracentrifuged again, it was resuspended with TBS (pH 7.4) containing 0.1% SB3-14. The supernatant of normal brain preparation after ultracentrifugation was used as the PrP^{C}-containing fraction. Total proteins were quantified by the modified Lowry method (22).

Western Blot, Peptide N-Glycosidase F (PNGase F) Treatment, and Competitive Enzyme-linked Immunosorbent Assay (ELISA)—For Western blot, samples of equal protein concentration of PrP^{C}-containing fraction, PrP^{Sc}-enriched insoluble fraction, PK (25 μg/insoluble fraction extracted from 1-g tissues, 2 h at 37 °C)-treated fraction, and PNGase F-treated fraction were separated with 12% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with either R3 anti-AGE antiserum (1:4,000) preincubated with BSA or AGE-BSA, 6D12 anti-AGE IgG (0.2 μg), and anti-PrP antibodies (3F4; 1:20,000 or 78295; 1:5,000) in blocking solution (blocked with TBS containing 5% skim milk and 0.05% Tween-20). Each membrane was incubated with goat anti-rabbit or anti-mouse IgG conjugated with peroxidase. After blotting shown in Fig. 1, *A*, *C*, and *E*, each membrane was stripped with buffer (2 ml of 10% SDS, 420 μl of 1.5 M Tris (pH 6.7), 70 μl of mercaptoethanol, 7.5 ml of triple-distilled H_2O). Next, membranes were blocked with blocking solution and incubated with goat anti-rabbit or anti-mouse IgG conjugated with peroxidase and developed with SuperSignal West Pico (Pierce). These processes demonstrate both complete stripping and exclusion of endogenous peroxidase activity. Thereafter, membranes were reprobed with monoclonal 3F4 anti-PrP antibody (Fig. 1*B*) or polyclonal 78295 anti-PrP antibody (Fig. 1, *D* and *F*).

For PNGase F analysis, 100 μg of total protein of the insoluble fraction was resuspended in lysis buffer. After heating for 10 min at 94 °C, it was supplemented with 5 units of PNGase F (Roche Applied Science) and incubated for 36 h at 37 °C. The reaction was stopped by the addition of sample buffer and analyzed by Western blot and immunoprecipitation.

For competitive ELISA, each well was coated with total proteins (30 μg) of denatured PrP^{Sc}-enriched insoluble fraction of 263K scrapie-infected brain and blocked with 1% BSA in TBS. After washing with TBS containing 0.1% BSA and 0.05% Tween-20, AGE competitors including AGE-modified amino acids or AGE-modified PrP peptides were added to each well followed by R3 anti-AGE antiserum.

Immunoprecipitation—For the immunoprecipitation assay, PrP^{C}-containing fractions, and PrP^{Sc}-enriched insoluble fractions of 263K or 139H scrapie-infected and PNGase F-treated 263K-infected brains

were boiled to unblock epitopes and were then immunoprecipitated with either R3 anti-AGE antibody, 6D12 anti-AGE antibody, or 3F4 anti-PrP antibody (each antibody was first coated to the surface of tosyl-activated magnetic Dynabeads M-280 (Dynal Biotech) according to a procedure described by the manufacturer). The complexes were washed several times with 0.1 M sodium phosphate buffer (pH 7.4) using a magnet (Dynal MPC, Dynal Biotech). Complexes were subsequently eluted by boiling in the presence of sample buffer. For immunoprecipitation studies, supernatants (30 μg of protein) containing PrP^C prepared from normal hamster brain and insoluble fraction (2 μg of protein) enriched with PrP^{Sc} from 263K or 139H scrapie-infected brains were used as the positive controls.

Ascorbate Autoxidation—Metals present in the buffers used in this study have the potential to induce peroxidase activity in PrP^{Sc} and thereby yield false positives on Western blots. Ascorbate oxidation experiments were performed to assess the level of contaminating metals in the buffers used in these experiments. For this test, 0.1 M ascorbate stock solution was prepared using reagent grade ascorbic acid (Sigma) and deionized high purity water (Bio-Rad). 3.75 μl of 0.1 M ascorbate stock solution was added to a total volume of 3.0 ml of each solution to be tested (23). The levels of ascorbate autoxidation are shown by the percentage loss of absorbance at 15 min compared with the determination of initial absorbance at 265 nm.

Immunohistochemistry—Following perfusion, brains were immediately removed, cut into blocks, post-fixed in 4% paraformaldehyde for 12 h at 4 °C, rinsed with PBS, dehydrated with ethanol, and then embedded in paraffin (Oxford). Coronal sections of the brains (6-μm thick) were cut with a microtome. Immunohistochemical analysis was carried out using the ABC kit (Vector) by a modification of the avidin biotin-peroxidase method. Briefly, sections of the brains were deparaffinized with xylene, hydrated with graded ethanol, and then treated with 0.3% hydrogen peroxide in methyl alcohol to block endogenous peroxidase. The sections were treated with PK (10 μg/ml, 10 min) to remove PrP^C, exposed to 10% normal donkey serum for 1 h, and then incubated with one of the following: R3 anti-AGE antiserum (1:500), rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:800, Jackson Laboratories), anti-PrP antibody (3F4 or 78295); and incubation was performed overnight at 4 °C. The sections were sequentially treated with biotinylated anti-rabbit (or anti-mouse) immunoglobulin followed by addition of the avidin-biotin peroxidase complex, developed with diaminobenzidine-hydrogen peroxide solution (0.003% 3,3-diaminobenzidine and 0.03% hydrogen peroxide in 0.05 M Tris buffer), and counterstained with hematoxylin.

Antibodies—Two different anti-AGE antibodies were used in this study: 1) rabbit polyclonal anti-AGE antibody against AGE-BSA, which was produced in this study (R3); and 2) mouse monoclonal anti-AGE antibody raised against AGE-BSA (6D12, TransGenic Inc.), which has been selected as AGE-HSA-positive and BSA-negative and is termed CML-positive (20, 24). Two different anti-PrP antibodies were used; mouse monoclonal anti-PrP antibody (3F4) (25) and rabbit polyclonal anti-PrP antibody (78295) (26). Anti-GFAP antibody (Jackson Laboratories) was used for GFAP in immunohistochemistry.

RESULTS

AGE-modified Proteins Accumulate in TSE Brains—We hypothesized that the long lasting PrP^{Sc} molecules would be the most likely material to assay for nonenzymatic glycation in the brains of TSE-infected animals. The anti-AGE-BSA antiserum was pre-adsorbed with BSA to ensure specific staining of AGE-modified protein. R3 anti-AGE antiserum raised against many different types of AGE epitopes reveals that AGE-modified proteins are at a high concentration in 263K and 139H scrapie-infected brains but not in controls (Fig. 1*A*). After stripping the membrane of R3 anti-AGE antibodies, PrP^{Sc} was detected with PrP^{Sc}-specific antibody, and these bands were identical to those seen with the AGE-antibody immunostaining (Fig. 1*B*). Following PK digestion, which removes ~90 amino acids from the N terminus of PrP^{Sc}, there was no detectable R3 anti-AGE antibody immunostaining (Fig. 1, *A* and *B*). AGE-modified proteins were also detected in mouse brains infected with the 139A, ME7, 22L, and 87V scrapie strains, and in the brains of CJD and vCJD patients; but in each case reactivity disappeared after PK digestion (Figs. 1, *C* and *E*, and 2*A*). However, PK-resistant prion isoforms were detected after PK digestion (Fig. 1, *D* and *F*). As indicated from the results with normal brain material, the presence of soluble PrP^C does not contribute to the results obtained for the insoluble fraction found in TSE brains (Figs. 1 and 2*A*). In the vCJD preparation, the lightly stained band with the lowest molecular mass (Fig 2*A*, *lane 5*) probably represents a small fragment of one of the PrP^{Sc} isoforms that has been modified with AGE. Preimmune serum did not detect AGE-modified proteins in PrP^{Sc}-enriched fractions nor did anti-AGE antiserum that had been preabsorbed with AGE-modified BSA. In contrast, preincubation of anti-AGE antiserum with BSA that had not been AGE-modified did not reduce AGE immunostaining to yield the same as Fig. 1, *A*, *C*, and *E* (data not shown). In addition, AGE-modified proteins were detected with 6D12 anti-AGE antibody in 263K-infected brain; there was minimal staining of an unknown protein with molecular mass of ~32 kDa in the brains of normal hamsters (Fig. 2*B*, *lane 1*). There were no immunoprecipitates obtained from control brain preparation supernatants containing soluble PrP^C (Fig. 2*E*). These results indicate that AGE-modified proteins accumulated only in TSE brains and are composed of protease-resistant PrP^{Sc}.

PrP^{Sc}, but Not PrP^C, Is Modified with AGE—Immunoprecipitation assays support the finding that PrP^{Sc} is nonenzymatically glycated in brain, but PrP^C is not glycated (Fig. 2, *C–F*). AGE-modified proteins were immunoprecipitated from 263K and 139H scrapie-infected brains, and then detected with 3F4 anti-PrP antibody (Fig. 2*C*). Conversely, prion isoforms were immunoprecipitated with 3F4 antibody and then detected with R3 anti-AGE antibody (Fig. 2*D*). Soluble PrP^C in the supernatant, however, was not immunoprecipitated, indicating that PrP^C is not modified with AGE (Fig. 2*E*). PNGase F was used to treat the insoluble fraction to remove one or both *N*-glycans of prion isoforms and then immunoprecipitated with 6D12 anti-AGE IgG. Deglycosylated isoform of AGE-modified prion proteins was detected with R3 anti-AGE antibody (Fig. 2*F*, *lane 1* and *2*). Moreover, three AGE-modified prion isoforms were found in PNGase F-untreated insoluble fractions after immunoprecipitation with 6D12 anti-AGE IgG (Fig. 2*F*, *lane 3*), although 6D12 does not immunoreact with the nonglycosylated isoform in Western blot (Fig. 2*B*, *lane 2*). This indicates greater sensitivity of immunoprecipitation compared with Western analysis.

Disease-associated Prion Is Modified with AGE at the Late Stage of Disease—PrP^{Sc} was detected at an early stage of disease in 263K-infected hamsters, even at 10 d.p.i., and then its accumulation increased throughout the clinical course of the disease. AGE modification of PrP^{Sc} was not detected until ~50 d.p.i. (Fig. 3, *A* and *B*). These results suggest that AGE modification occurs postconversion of PrP into the PrP^{Sc} conformation; however, it should be kept in mind that this result could be a function of the relative sensitivities of the antibodies, rather than a reflection of the timing of events.

The issue of possible nonspecific endogenous peroxidase activity yielding false positive staining was of some concern. In fact, using the ascorbate test for catalytic metals (Buettner, Ref. 23) that could bind to PrP^{Sc} and thereby produce endogenous peroxidase activity, we found that several buffers (0.1 M and 0.5 M sodium phosphate buffer, TBS, TBS containing 20% *N*-lauroyl sarcosine, TBS containing 10% NaCl and 0.1% SB3-14, and TBS containing 0.1% SB3-14) contained catalytic metals. Those buffers that included SB3-14 were free of metals (Table II). To rule out the potential that AGE-modified PrP^{Sc} possesses endogenous peroxidase activity, a membrane was developed with SuperSignal West Pico including peroxide and enhancer solution without incubation with antibody. This blot showed that AGE-modified PrP^{Sc} did not possess endogenous

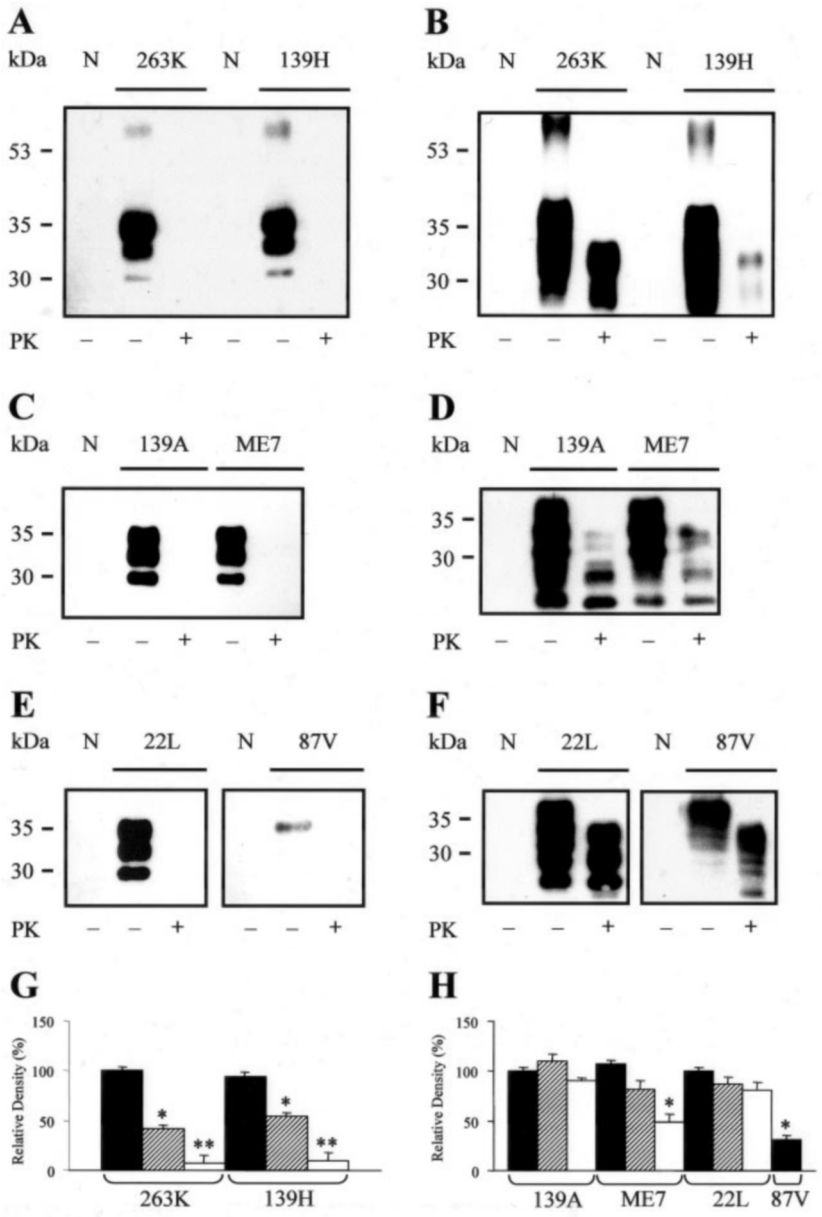

FIG. 1. **Accumulation of AGE-modified proteins in PrP^{Sc}-enriched insoluble fractions of scrapie-infected brains.** Normal brains (*N*) and scrapie-infected brains (*A* and *B*, 263K and 139H; *C* and *D*, 139A and ME7; *E* and *F*, 22L and 87V) were homogenized and then ultracentrifuged as described under "Experimental Procedures." Total proteins (15 μg of the insoluble fraction) in *A*, *C*, and *E* were loaded and then blotted with R3 anti-AGE antiserum. After the membranes were examined in *A*, *C*, and *E*, they were stripped and reprobed with monoclonal 3F4 anti-PrP antibody (*B*) or with polyclonal anti-PrP antibody (*D* and *F*). Molecular mass is indicated at the *left side* of each figure. *G* and *H*, the blots in *A*, *C*, and *E* were quantified by scanning densitometry, and the values are shown in *G* and *H*. Data are expressed as mean ± S.D. of three independent experiments. **, $p < 0.01$; *, $p < 0.05$ compared with highest AGE-modified protein (*black bar*) blotted in 263K, 139A, and 22L as assessed by Student's *t* test. Middle AGE-modified protein, *diagonal bar*; lowest AGE-modified protein, *white bar*.

peroxidase activity (Fig. 2*B*, *4 lanes* on *right portion of blot*). Furthermore, in Fig. 3*B*, PrP^{Sc} is present at 40 d.p.i., but this sample does not reveal positive staining in the anti-AGE blot (Fig. 3*A*), indicating that PrP^{Sc} does not have endogenous peroxidase activity. Thus, despite the presence of contaminating metals in some of the buffers used in these studies (Table II), there was no evidence that the PrP^{Sc} assayed by Western blots contained endogenous peroxidase activity.

Characteristics of AGE Modification of PrP^{Sc}—There are several key points with regard to the appearance patterns of AGE-modified PrP^{Sc}. First, in 263K- and 139H-infected hamsters, AGE-mediated modification occurs most often in diglycosylated PrP^{Sc} and least often in nonglycosylated PrP^{Sc}. In contrast, AGE modification is distributed differently in most scrapie strain-mouse strain combinations (Fig. 1, *A* and *C* and *22L* in *E*, *G*, and *H*). In the 87V-infected brain, for example, only diglycosylated PrP^{Sc} appears to be modified with AGEs (Fig. 1, *E* and *H*). It is possible that the differences in AGE modifications of PrP^{Sc} are a function of the TSE strain host combination (27, 28). Second, identification of AGE modification of nonglycosylated PrP^{Sc} indicates that the epitopes are not *N*-glycans but AGE products of nonenzymatic glycation of the protein component of PrP^{Sc} (Fig. 1, *A* and *C* and *22L* in *E*). This result is supported by PNGase F analysis (Fig. 2*F*, *lanes 1* and *2*).

Nonenzymatic AGE Modification of PrP^{Sc} Occurs at the N-terminal Lys Residue and May Be at Arg^{37}—We speculated that the AGE epitope(s) is within the residues 23–89 of prion protein, because it was not detected in PK-digested fractions (Fig. 1). In order to map the epitope(s) involved in AGE-modified PrP^{Sc}, we nonenzymatically glycated PrP peptides occurring at the N terminus, specifically PrP peptide 23–36 and PrP peptide 37–50, incubated for 50 days. As shown in Fig. 4*A*, *in vitro* glycoxidation produces R3-positive AGE-mediated modification of BSA molecules (Fig. 4*A*, *lane 1*) and PrP peptide 23–36 molecules (Fig. 4*A*, *lane 3*) following AGE modification, but does not yield R3-positive AGE-PrP peptide 37–50 after incubation for 50 days. Both AGE-PrP peptide 23–36 and AGE-BSA are capable of competing with AGE-modified PrP^{Sc} as shown by competitive ELISA (Fig. 4*B*), whereas AGE-PrP peptide 37–50 incubated for 50 days is not. These results indicate that the extreme N terminus of PrP^{Sc} can be post-translationally modified through AGE-mediated modification and suggest that AGEs formed from PrP peptide 23–36 are the nonenzy-

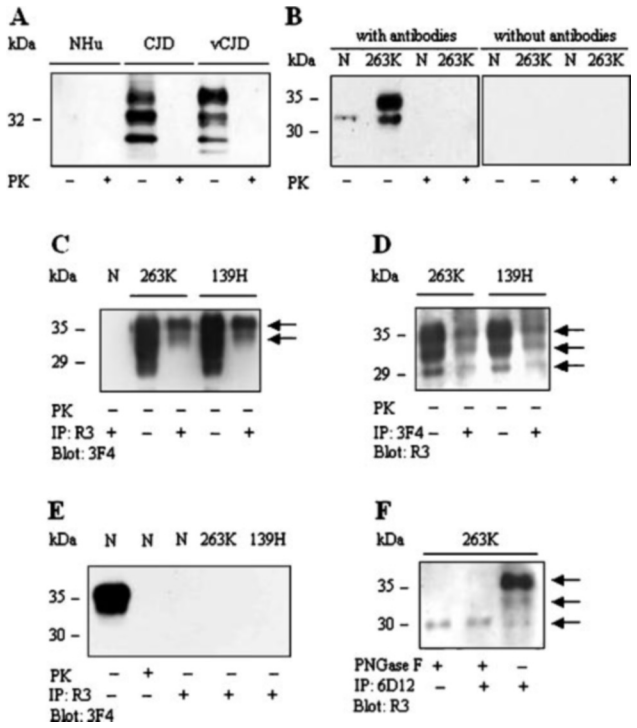

FIG. 2. **AGE modification of disease-associated prion isoforms.** 10 μl of the insoluble fraction from brains of normal human (*NHu*), CJD, and vCJD (*A*), and total proteins (15 μg of the insoluble fraction) from normal and 263K-infected brain (*B*) were loaded and then blotted with R3 anti-AGE antiserum (*A*), or with monoclonal 6D12 anti-AGE antibody (*4 left lanes* in *B*), or developed with SuperSignal West Pico without incubation with antibody (*4 right lanes* in *B*). Normal brains (*N*) and scrapie brains (263K and 139H) were homogenized, ultracentrifuged, and divided into insoluble (*C*, *D*, and *F*) and supernatant (*E*) fractions, which were used for immunoprecipitation with R3 anti-AGE antibody (*C* and *E*), or with 3F4 anti-PrP antibody (*D*), or with monoclonal 6D12 anti-AGE antibody (*F*). *C* and *E* were blotted with the 3F4 anti-PrP antibody. *D* and *F* were blotted with R3 anti-AGE antibody. *Arrows* in *C*, *D*, and *F* indicate the prion isoforms (*C*) and AGE-modified prion isoforms (*D* and *F*), respectively.

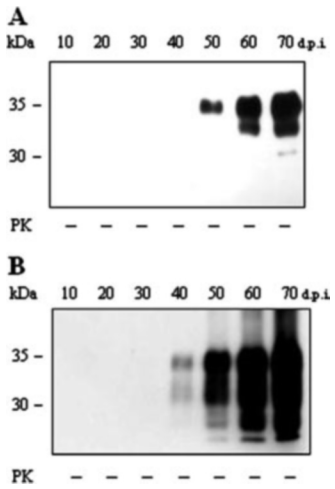

FIG. 3. **Kinetics of AGE modification of prion isoforms.** Nonenzymatic AGE modification of disease-associated prion isoforms occurs at the late stage of disease but does not occur at the preclinical stage. *A*, 263K-infected brains were isolated at 10-day intervals. For each lane, a total of 15 μg of PrP^{Sc}-enriched insoluble fraction was loaded and then blotted with R3 anti-AGE antiserum. *B*, after the membrane used in *A* was stripped, it was blotted with the 3F4 anti-PrP antibody. Molecular mass is indicated at the *left side* of each figure.

matically glycated component in PrP^{Sc} found *in vivo*. As noted previously, AGE modification occurs predominantly at the amino groups in the side chains of amino acids such as lysine and arginine of long lived proteins (29). To assess which amino acids are associated with AGE formation at the N terminus of PrP^{Sc}, we incubated D-glucose with lysine and arginine, at different ratios of D-glucose and the amino acids and at several days of incubation. The R3 anti-AGE antibody raised against AGE-BSA did not react with AGE-Lys or AGE-Arg that formed after 7 days of incubation (Fig. 4*A*), indicating that the AGE-modified portion of peptide 23–36 is different from the Lys-derived AGE or Arg-derived AGE epitopes. The arginine- and lysine-rich region of PrP 23–36, residues 23–28, were not glycated with glucose *in vitro*. Neither did R3 anti-AGE antibody react with any glycated amino acids produced *in vitro* (data not shown).

To determine the exact AGE epitope in the N-terminal region of AGE-modified PrP^{Sc}, we synthesized a series of 14-mer PrP peptides, starting from Lys^{23} and continuing to residue 37. These peptides were then incubated with D-glucose for 90 days (Table I). AGE-modified PrP peptides were stained with Coomassie Brillant Blue (Fig. 5*A*), and a second gel was blotted with anti-AGE antibody R3 (Fig. 5*B*). The levels of staining vary significantly (Fig. 5*A*) according to the composition of amino acids (Table I). AGE-Lys and AGE-Arg incubated for 50 days were also stained, but were not positively blotted with R3 antibody (Fig. 5*B*). AGE-PrP peptides that started from Lys^{23} (*lane 1*), Lys^{24} (*lane 2*), and Pro^{26} (*lane 4*) were strongly immunostained with R3 compared with AGE-PrP peptides that started from Lys^{27} (*lane 5*) and Arg^{37} (*lane 15*) (Fig. 5*B*). However, residue 25 that starts from Arg was not positively stained with R3 anti-AGE antibody (Fig. 5*B*, *lane 3*). We obtained similar results using the 6D12 antibody (data not shown), except that only R3 immunoreacted with the peptide that starts with residue 37 (Fig. 5*B*, *lane 15*), indicating that a positive reaction with this peptide depends on the length of incubation of the peptide with D-glucose (compare the staining in Fig 5*B*, *lane 15* after 90 days of incubation with Fig. 4*A*, *lane 5* after 50 days of incubation). The results also suggest that the polyclonal R3 antibody has a wider range of epitope reactivity than the 6D12 monoclonal antibody. 6D12 positive reactivity to AGE-modified PrP peptides 1, 2, 4, and 5 suggests that the N-terminal lysine residues are modified with CML because CML is a major target of 6D12 immunoreactivity (20, 24). There were no immunoreactions with anti-AGE antibodies to the same 15 peptides incubated for the same periods in the absence of D-glucose (data not shown). These results show that the variation of the level of nonenzymatic glycation depends on the length of the incubation period and on the composition of amino acids in N-terminal PrP residues.

The immunoreactivity of two anti-AGE antibodies (R3 and 6D12) to AGE-PrP peptides that start at residues 23, 24, and 26 can be explained by the lysines in those three peptides. The failure to obtain reactivity with peptide 3, which also contains lysine, suggests that either the epitope in AGE-modified peptide 3 was not conclusively reactive to antibody or that the configuration of the peptide prevented AGE formation. The reactivity of the R3 antibody with peptide 15 argues that the arginine in that peptide was AGE-modified and that the configuration was immunoreactive. Competitive ELISA supports these results. The competitions corresponded to the level of immunoreactivity of R3 anti-AGE antibody to AGE-PrP peptides (Fig. 5*D*), whereas non-AGE-modified PrP peptides did not compete with AGE-modified PrP^{Sc} (Fig. 5*C*).

AGEs Localize in Astrocytes in 263K and 87V Scrapie-infected Brain—Cellular localization of AGE-modified PrP^{Sc} was evaluated by immunohistochemical methods. AGEs and GFAP were immunostained with their respective R3 anti-AGE (Fig.

TABLE II
Ascorbate autoxidation test

As indicated under "Experimental Procedures," 3.75 μl of 0.1 M ascorbate stock solution was added to 3.0 ml of each solution to be tested. The levels of ascorbate autoxidation are shown by the percentage loss of absorbance (*A*) at 15 min compared to the determination of initial absorbance at 265 nm (% loss of $A = A$ at 15 min/A at 0 min) × 100). Data are expressed as a mean ± S.D. of three independent experiments.

Solutions	Deionized pure water	A[a]	B[b]	C[c]	D[d]	E[e]	F[f]
% Loss of *A*	72.6 ± 2.4	51.9 ± 3.2	49.2 ± 2.9	37.1 ± 3.6	24.3 ± 3.8	95.6 ± 2.2	97.7 ± 1.4

[a] Solution A: 100 mM sodium phosphate buffer.
[b] Solution B: 500 mM sodium phosphate buffer.
[c] Solution C: TBS, pH 7.4.
[d] Solution D: TBS, pH 7.4 containing 20% *N*-lauroyl sarcosine.
[e] Solution E: TBS, pH 7.4 containing 0.1% SB3–14.
[f] Solution F: TBS, pH 7.4 containing 10% NaCl and 0.1% SB3–14.

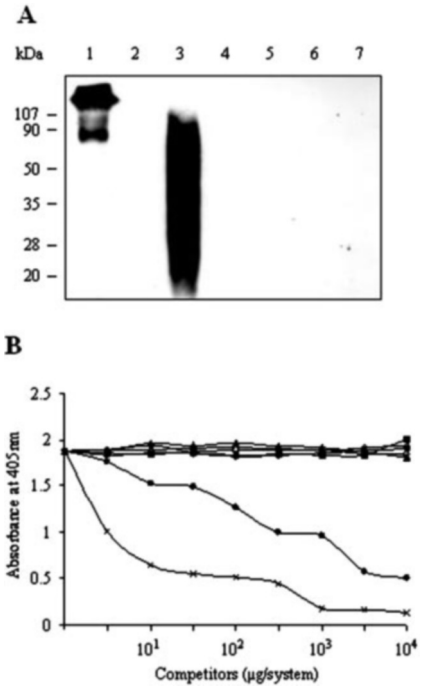

FIG. 4. **Analysis of AGE modification at several N-terminal sequences of PrP^{Sc} (23–231).** *A*, each lane was blotted with R3 anti-AGE antiserum. AGE-modified BSA, 1 μg (*lane 1*). From *lanes 2–7* each lane received 100 μg: native PrP peptide 23–36 (*lane 2*), native PrP peptide 37–50 (*lane 4*), AGE-modified PrP peptides (AGE-PrP peptide 23–36, *lane 3* and AGE-PrP peptide 37–50, *lane 5*). All of the above preparations were incubated for 50 days except for AGE-BSA incubated for 90 days. AGE-modified lysine (AGE-Lys, *lane 6*) and AGE-modified arginine (AGE-Arg, *lane 7*) were incubated for 7 days. Molecular mass is indicated at the *left side* of the figure. *B*, competitive ELISA. The following peptides were used as competitors of AGE-modified PrP^{Sc}: native PrP peptide 23–36 (*open circle*), native PrP peptide 37–50 (*open triangle*), AGE-PrP peptide 23–36 (*closed circle*), AGE-PrP peptide 37–50 (*closed triangle*), AGE-K (*closed diamond*), AGE-R (*closed square*), and AGE-BSA (*multiplication sign*). Data are expressed as a mean of five independent experiments.

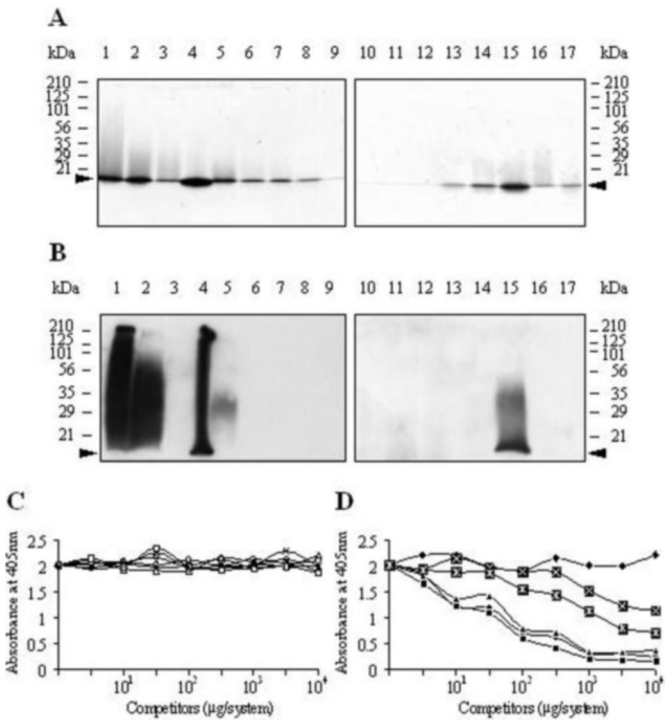

FIG. 5. **N-terminal Lys residues of prion peptide are reacted with anti-AGE R3 antibody.** AGE-modified PrP peptides (500 μg) were loaded and Coomassie Brilliant Blue-stained (*A*) or blotted with R3 anti-AGE antibody (*B*). *Lanes 1–15* correspond to AGE-modified products of PrP peptides as shown in Table I; these 14-mer peptides were incubated with D-glucose for 90 days. *Lanes 16* and *17*, AGE-Lys and AGE-Arg, respectively, were incubated for 50 days. *Arrowheads* indicate the end of electrophoresis. *C* and *D*, competitive ELISA. The following peptides were used as competitors of AGE-modified PrP^{Sc}: *C*, peptides were not exposed to D-glucose: PrP peptide 1 (*open circle*), PrP peptide 2 (*open triangle*), PrP peptide 3 (*open diamond*), PrP peptide 4 (*open square*), PrP peptide 5 (*multiplication sign*), and PrP peptide 15 (*asterisk*) in Table I. *D*, peptides were incubated with D-glucose. PrP peptide 1 (*closed circle*), PrP peptide 2 (*closed triangle*), PrP peptide 3 (*closed diamond*), PrP peptide 4 (*closed square*), PrP peptide 5 (× *with black background*), and PrP peptide 15 (*asterisk with black background*). Data are expressed as a mean of three independent experiments.

6*C*) and anti-GFAP (Fig. 6*D*) antibodies in the hippocampus of 263K scrapie-infected brain, whereas there was virtually no staining for either AGE-modified protein (Fig. 6*A*) or reactive astrocytes (Fig. 6*B*) in control hamster brain. Similar results were also observed in the cerebral cortex (data not shown). In addition, AGEs primarily localized in the reactive astrocytes of 263K-infected brain and colocalize with PK-resistant isoform of the prion protein as shown by serial immunostaining for AGEs and GFAP (Fig. 6, *C* and *D*), and for AGEs and PK-digested PrP isoform (Fig. 6, *E* and *F*), respectively. AGE staining was weaker than that seen in astrocytes in brains of 87V mice (Fig. 6, *G* and *H*). A notable aspect in immunostaining for GFAP, AGEs, and PrP^{Sc} is that all of the GFAP-positive astrocytes are not AGE-positive, whereas it seems likely that most of the AGE-positive astrocytes are PrP^{Sc}-positive, intimating that AGEs may be an additive risk factor for prion replication or prion deposition (Fig. 6, *E* and *F* and *G* and *H*).

DISCUSSION

Although the roles of normal and disease-associated prion isoforms in the infection process remain unclear, their importance in the pathogenesis of TSEs has been well documented (30–32). The findings in the current study clearly indicate that PrP^{Sc} is modified by AGEs, characterized in large part by CML. This modification was seen with all of the TSE examined: 1) four mouse-adapted scrapie strains (ME7, 139A, 22L, and 87V),

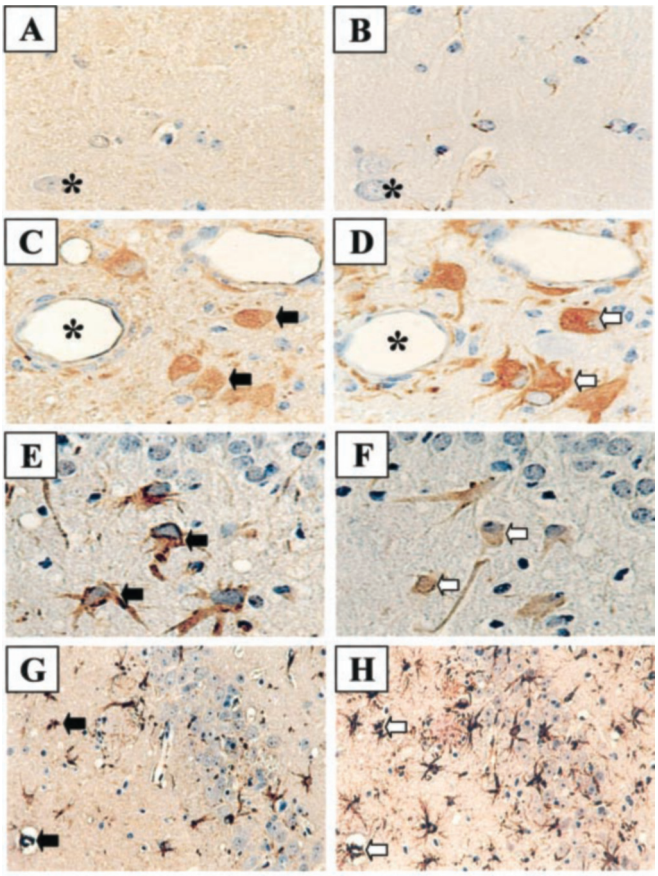

FIG. 6. **Cellular localization of AGEs in 263K and 87V scrapie-infected brain.** PrP^{Sc} appears to be nonenzymatically glycated in astrocytes. Each pair of the following are serial sections: *A* and *B*, *C* and *D*, *E* and *F*, *G* and *H*. Normal hamster brain showed little or no immunostaining with antibodies to AGEs (*A*, R3) or to GFAP (*B*). For 263K scrapie-infected brain, AGE-positive cells (*C*, *black arrows*) colocalize with GFAP-positive astrocytes (*D*, *white arrows*). Also, AGEs (*E*, *black arrows*) colocalize with PK-digested prion isoform (*F*, *white arrows*) in the same cells of 263K scrapie-infected brain. For 87V scrapie-infected brain, some of the GFAP-positive astrocytes (*H*, *white arrows*) also stain with AGE antibody (*G*, *black arrows*). *Asterisks* in *A* and *B*, and *C* and *D* represent landmarks for serial sections. *A–F*, ×200, *G* and *H*, ×400.

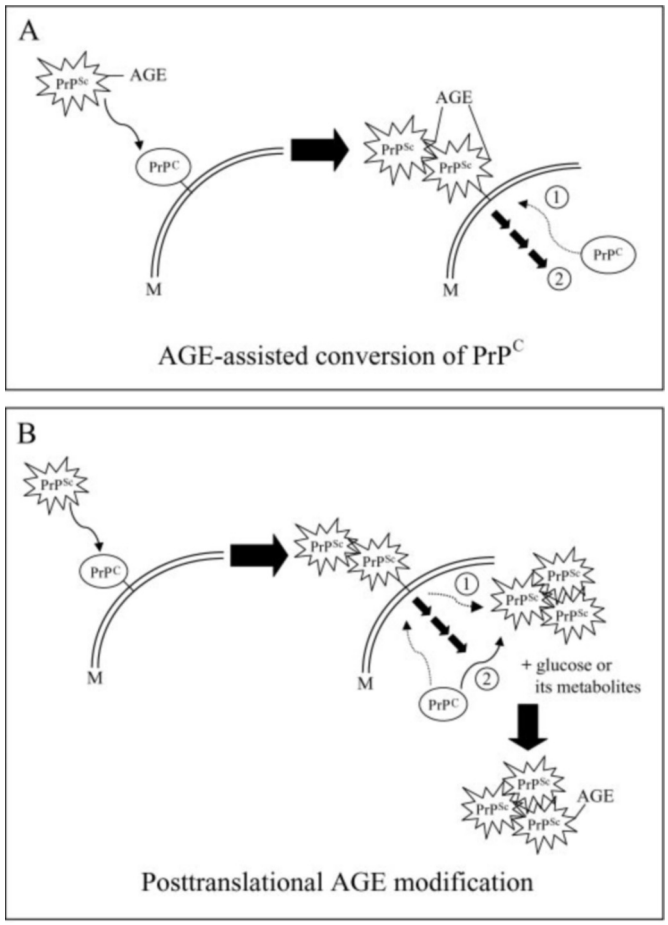

FIG. 7. **Possible scenarios of prion conversion and polymerization by AGE modification.** *A*, AGEs assist in prion conversion. The N terminus of long lived PrP^{Sc} is nonenzymatically glycated with glucose or its metabolites, and is then modified to form AGEs. This AGE-modified PrP^{Sc} interacts with PrP^{C} through covalent AGE modification and subsequently initiates the conformational conversion of PrP^{C}. Continuous conversion will require either that PrP^{C} is transported to the plasma membrane for glycophosphatidylinositol-anchoring (*1*) or that raft-associated PrP^{Sc} is processed in the endocytic route to interact with cytoplasmic PrP^{C} (*2*). *B*, AGEs affect pre-existing PrP^{Sc}. Nonenzymatic glycation at the N terminus of PrP^{Sc} may occur after the conversion (and then act, at least in part, as an additive risk factor that enhances PrP^{Sc} stability). *M*, plasma membrane.

2) two hamster-adapted scrapie strains (263K and 139H), and 3) both sporadic CJD and vCJD.

The AGE modification became evident late in the incubation period of the disease and appeared to occur ~40 days after the first evidence of PrP^{Sc}. In Fig. 3*B*, PrP^{Sc}-positive immunostaining was not seen until 40 days post-infection; however, further exposure of the blots revealed PrP^{Sc} staining as early as 10 days after infection (data not shown). It should be noted, however, that the difference in time of appearance of the protein and of its glycation could be a consequence of sensitivity differences among antibodies. A major site of AGE modification was located at the PK-sensitive N terminus of PrP^{Sc}, as shown by the fact that there were no positive reactions in PK-treated insoluble fractions using the polyclonal R3 antibody or the monoclonal 6D12 antibody, each of which reacted with non-PK-treated scrapie and CJD preparation. More specifically, the AGE modification occurred between peptides 23–36, as confirmed by competition studies in which AGE-modified PrP peptide 23–36 reduced the immunoreactivity of anti-AGE antibody to PrP^{Sc}. In contrast, neither AGE-modified PrP 37–50 (incubated for 50 days) nor several other AGE products associated with the following (33): lysine, arginine, and their combinations, were able to compete with the anti-AGE antibodies used in this study. The results from a series of AGE-modified 14-mer PrP peptides, which ranged from residue 23 to 50, show that one or more residues Lys^{23}, Lys^{24}, and Lys^{27} of PrP^{Sc} are modified with AGE. In addition, residue Arg^{37} may also be modified with AGE because it is positive to R3 anti-AGE antibody after incubation with D-glucose for 90 days.

One of the pathophysiological mechanism(s) that lead to the formation of PrP^{Sc} fibrils in TSE-affected brain may be stabilized and strengthened by the progression of specific AGE modification of PrP^{Sc}. Both R3 anti-AGEs antibody and 3F4 anti-PrP antibody reacted with protein bands with a molecular mass of ~55 kDa (Fig. 1, *A* and *B*). The structure of AGE-mediated modification of BSA and PrP-(23–36) can be maintained during SDS-PAGE analysis (Fig. 4*A*, *lanes 1* and *3*, Fig. 5*B*, *lanes 1*, *2*, *4*, *5*, and *15*). In this regard, the modification by AGE may be responsible for the dimerization of PrP^{Sc} and further polymerization (34).

It has been shown previously that PrP^{Sc} is first detected in astrocytes in scrapie-infected mice (35). In the current study, AGE-modified PrP^{Sc} was found in GFAP-positive cells. Not all of the GFAP-positive cells were immunostained for AGEs, but most of the AGE-positive cells contained PrP^{Sc}. This finding

supports the concept that the abnormal prion protein is a major target for glycation in the TSEs.

The role of AGE in the formation of PrP^{Sc} is not known at this time. There are two possibilities that are outlined in Fig. 7. In Fig. 7*A*, all AGE modification occurs at the time of PrP^{Sc} formation, and the glycation process may assist in the production of PrP^{Sc}. The covalent attachment of AGEs to PrP^{Sc} may trigger the conversion of PrP^{C} to PrP^{Sc} or the process could be accelerated either by the glycation of the infecting PrP^{Sc} or a direct effect on the PrP^{C}-PrP^{Sc} complex at the time of formation. A second scenario is shown in Fig. 7*B*. Glycation occurs after the formation of PrP^{Sc}, perhaps long after its conversion from PrP^{C}. Glycation at this point could still play a role in the disease process, because the glycation would provide added protection from cellular degradation for the PrP^{Sc} molecules *in vivo*. At present, there is insufficient data to distinguish these two possible scenarios. The finding that PrP^{Sc} could be detected prior to the appearance of AGE positivity (Fig. 3, *A* and *B*) can be viewed as support for the second scenario (Fig. 7*B*); however, this time interval with regard to positive findings could be a function of differences in the avidity and specificity of the detection antibodies. The findings that AGE immunohistostaining is seen in astrocytes, which have been reported to be the first cell type that develops PrP^{Sc}. This combined with the fact that most cells that are PrP^{Sc}-positive are also positive for AGEs immunostaining (Fig. 6, *E* and *F*) would support the first scenario (Fig. 7*A*) (35). More experiments will be required to determine the role of PrP^{Sc} glycation in the disease process.

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