Gerstmann-Sträussler-Scheinker Disease and “Anchorless Prion Protein” Mice Share Prion Conformational Properties Diverging from Sporadic Creutzfeldt-Jakob Disease*

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Background: Prion strains exhibit distinct physical and biochemical repertoires, aggregation propensity, and biological properties. Results: A biochemical approach is developed for defining the conformational features of prions with or without glycosylphosphatidylinositol (GPI)-anchor. Conclusion: GPI anchorless prions are detected in human genetic prion diseases, but not in sporadic forms. Significance: Unveiling the structure of GPI anchorless prions to predict pathological properties.

The role of the GPI-anchor in prion disease pathogenesis is still a challenging issue. In vitro studies have shown that anchorless cellular prion protein (PrPC) undergoes aberrant post-translational processing and metabolism. Moreover, transgenic (Tg) mice overexpressing anchorless PrPC develop a spontaneous neurological disease accompanied with widespread brain PrP amyloid deposition, in the absence of spongiform changes. Generation of PrP forms lacking the GPI and PrP amyloidosis are striking features of human stop codon mutations in the PrP gene (PRNP), associated with PrP cerebral amyloid angiopathy (PrP-CAA) and Gerstmann-Sträussler-Scheinker (GSS) syndrome. More recently, the presence of anchorless PrP species has been also claimed in sporadic Creutzfeldt-Jakob disease (sCJD). Using a highly sensitive protein separation technique and taking advantage of reference maps of synthetic PrP peptides, we investigated brain tissues from scrapie-infected “anchorless PrP” Tg mice and wild type mice to determine the contribution of the GPI-anchor to the molecular mass and isoelectric point of PrP quasispecies under two-dimensional electrophoresis. We also assessed the conformational properties of anchorless and anchored prions under standard and inactivating conditions. These studies were extended to sCJD and GSS. At variance with GSS, characterization of PrP quasispecies in different sCJD subtypes ruled out the presence of anchorless prions. Moreover, under inactivating conditions, mice anchorless prions, but not sCJD prions, generated internal PrP fragments, cleaved at both N and C termini, similar to those found in PrP-CAA and GSS brain tissues. These findings show that anchorless PrPSc generates GSS-like PrP fragments, and suggest a major role for unanchored PrP in amyloidogenesis.

Transmissible spongiform encephalopathies (TSEs), 3 or prion diseases, include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and GSS in humans, chronic wasting disease in cervids, scrapie in sheep, and bovine spongiform encephalopathy in cattle (1).

In TSEs, PrPC is converted to an abnormal isoform, or PrPSc, characterized by relative resistance to protease digestion and detergent insolubility (2). PrPC is a GPI-anchored glycoprotein, which in its mature form spans residues 23–231 after the removal of the N-terminal and C-terminal signal sequences, and attachment of the GPI-anchor at residue 231 (3, 4). Post-translational PrPSc processing involves endosomal recycling of the cell surface full-length protein and proteolytic cleavages at residues 111/112 and ~90, leading to the generation of N-terminally truncated C1 and C2 fragments (5); an additional proteolytic cleavage of PrPSc is thought to occur at the C terminus, near the GPI-anchor, which results in detachment of the full-length protein from the cellular surface. Even though the bulk of brain PrPSc is GPI-anchored, protease-mediated shedding of the PrPSc ectodomain or enzymatic cleavage of the GPI phospholipid moiety have been reported in the cerebrospinal fluid (CSF), plasma, urine, and cell cultures (6, 7, 8). However, physiologically shed PrPSc species display distinct features from PrPC experimentally exposed to the enzyme phosphatidylinositol-

The abbreviations used are: TSEs, transmissible spongiform encephalopathies; BH, brain homogenates; sCJD, sporadic Creutzfeldt-Jakob disease; GSS, Gerstmann-Sträussler-Scheinker syndrome; PRNP, human prion protein gene; PrPc, cellular prion protein; PrPSc, disease-associated PrP; GPI, glycosylphosphatidylinositol moiety; GPI-Tg mice, GSS negative transgenic mice; PK, proteinase-K.

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specific phospholipase C (PI-PLC). Indeed, upon delipidation of PrP\textsuperscript{C} molecules with PI-PLC, the diacylglycerol hydrophobic tail is hydrolyzed, with exposure of negatively charged phosphate groups by the retained portion of the anchor. The detachment of the phospholipid moiety accounts for the paradoxical slower migration of PI-PLC-digested PrP\textsuperscript{C} species under SDS-polyacrylamide gel electrophoresis (9). Expectedly, this effect is not observed upon PI-PLC treatment of native and recombinant anchorless PrP\textsuperscript{C} molecules (10) and of post-translational modified PrP\textsuperscript{Sc} species lacking the phospholipid component (8). On the other hand, in vitro studies have shown that anchorless PrP\textsuperscript{C} is not tethered to the cell membrane, and is not recycled within the endosomal compartment, being instead secreted extracellularly (11).

Intriguingly, transgenic mice overexpressing anchorless PrP\textsuperscript{C} develop a spontaneous GSS-like neurologic illness with widespread PrP amyloid deposition in brain tissues, as a result of aggregation and accumulation of an internal PrP fragment (12). Additionally, infection of “anchorless PrP” mice induces the formation of angiocentric amyloid plaques, as opposed to granular PrP\textsuperscript{Sc} deposition observed in wild-type mice (13). This suggests that GPI-anchored and anchorless PrP are converted at diverse subcellular and/or extracellular sites, and spread through different routes (14). Anchorless and anchored PrP\textsuperscript{Sc} molecules display marked variability and heterogeneity in their glycosylation profile, generation of protease-resistant quasispecies, and aggregation propensity, adding further complexity to their physicochemical features and strain properties. Although valuable information has been gained from experimental models, a clear definition of molecular properties of anchorless PrP in human prion disorders is still missing.

sCJD, the most common human prion disease, has an annual incidence of ~1.5–2 per million worldwide, and a still unknown etiology. Prevailing hypotheses suggest that the disorder is triggered by spontaneous changes in PrP\textsuperscript{Sc} conformation, although concern has been raised that some sCJD cases might occur as a consequence of environmental exposure, case-to-case transmission, or food contamination (15). On a molecular ground, sCJD PrP\textsuperscript{Sc} is characterized by two major types of PrP27–30 with unglycosylated peptides of 21 and 19 kDa, in addition to distinct C-terminal fragments, but not internal PrP truncated fragments.

Recently, the generation of anchorless PrP forms has been also claimed in sCJD, hence suggesting that in addition to anchored PrP\textsuperscript{Sc} conformers, anchorless molecules could contribute to the phenotypic heterogeneity of this disorder (16). This issue raises additional concerns regarding the neuroinvasive properties of these quasispecies and the potential infectivity of human body fluids and peripheral tissues of sCJD patients. In the present study, we used a highly sensitive protein separation technique to assess the electrophoretic coordinates of anchorless and anchored PrP\textsuperscript{Sc} isoforms, using a panel of different synthetic PrP peptides as a reference map. Further, we exploited the conformational properties of anchorless and anchored prions to investigate their expression in different sCJD subtypes.

**Experimental Procedures**

**Synthetic Peptides and Antibodies**—Human synthetic PrP peptides spanning sequences 23–230, 90–230, 105–230, and 121–230 were purchased (Alcón AG, Zurich, CH); PrP peptide 82–146 was kindly donated by Dr. M. Salmona. The following mouse monoclonal antibodies recognizing different human PrP epitopes were used: 3F4, residues 108–111 (Signet Laboratories), 6D11, residues 93–109 (Signet Laboratories), ICSM-35, residues 93–102 (α-Gen UK), 12B2, residues 89–93 (kindly donated by Dr. J.P.M. Langeveld), SAF70, residues 142–160 (Cayman Chemicals), 6H4, residues 144–152 (Pronics, CH), 4G11, residues 199–216, and 3E2, residues 214–231 (kindly donated by Dr. L. Capucci).

**Animal Inoculation**—All mice were housed at the Rocky Mountain Laboratories (RML) in an AAALAC-accredited facility. Research protocols and experimentation were approved by the NIH RML Animal Care and Use Committee. Transgenic GPI anchorless PrP mice (tg444/+ α) were generated as previously described (13). Four to six-week-old mice were inoculated intracerebrally with 50 μl of a 1% brain homogenate of RML scrapie containing 0.7–1.0 × 10\textsuperscript{6} ID\textsubscript{50}. One ID\textsubscript{50} is the dose causing infection in 50% of C57BL/10SnJ mice. Animals were observed daily for onset and progression of scrapie. Mice were euthanized when clinical signs were consistent and progressive.

**sCJD Tissue Samples**—Brain samples were obtained from 29 cases of definite sCJD, 11 methionine homozygous at codon 129 with type 1 PrP\textsuperscript{Sc} (MV1), 3 methionine/valine with type 1 PrP\textsuperscript{Sc} (MV2), 7 MM with type 2 PrP\textsuperscript{Sc} (MM2), 5 MV2, 5 VV2, and 1 case of variant CJD (vCJD), diagnosed according to current criteria (17). Post-mortem intervals ranged from 4 to 30 h. Genomic DNA, extracted from frozen brain tissues, was searched for PrNP mutations and M/V polymorphism at codon 129. Neuropathological and immunohistochemical studies were performed as described previously (18).

**Immunoblot Analysis**—Brain samples were homogenized in 9 volumes of lysis buffer (100 mM sodium chloride, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.4). Aliquots were adjusted to a final concentration of 50 μg of proteinase K (Roche Applied Science, Germany) per milliliter and incubated at 37 °C for 60 min; protease digestion was quenched by adding PMSF to a final concentration of 2 mM (Boehringer, Mannheim). In some experiments, PMFS was omitted and samples were dissolved in Laemmli buffer (3% SDS, 3% β-mercaptoethanol, 2 mM EDTA, 10% glycerol, 62.5 mM Tris, pH 6.8), before boiling at 100 °C for 5 min. The combined exposure of samples to the denaturing anionic detergent SDS and PK, was exploited to solubilize non-amyloid PrP complexes and assess the conformation of PrB\textsuperscript{Sc} species under conditions altering the quaternary and tertiary structure of PrB\textsuperscript{Sc}.

For N-deglycosylation, samples were treated with N-glycosidase F (PNGase-F) according to the manufacturer’s instruction (Roche Applied Science) for 8 h at 37 °C. Samples were dissolved in Laemmli buffer and boiled for 5 min. An equivalent of 0.4 mg of wet tissue was loaded on 13% SDS-PAGE gels and proteins were transferred onto PVDF membrane (Immobilon P, Millipore) for 2 h at 60 V. Membranes were blocked with 1%
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nonfat dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at 37 °C and incubated overnight at 4 °C with anti-human PrP monoclonal antibodies (3F4, 1:5,000; 6D11, 1:3,000; ICSM-35, 1:1,000; 12B2, 1:8,000; SAF70, 1:1,000; 6H4, 1:5,000; 4G11, 1:1,000; 3E2, 1:3,000). Blots were developed with an enhanced chemiluminescence system (ECL, Amersham Biosciences) and PrP visualized on autoradiographic films (Hyperfilm, Amersham Biosciences). Films were scanned by using a densitometer (GS-200, Bio-Rad).

Two-Dimensional Gel Electrophoresis (2D-PAGE)—For isoelectric focusing (IEF), using immobilized pH gradients (IPG) in the first dimension, pre-cast gels with a linear pH range of 3–10 were used (Bio-Rad). Before IEF, the dry gels were reswollen for 14–15 h in 125 μl of buffer (6 mM urea, 2 mM thiourea, 5% β-mercaptoethanol, 2% Nonidet P-40, and 2% Ampholytes) containing the equivalent of 2 mg of wet tissue. IEF was carried out at 20 °C for 4 h with raising voltage (500–8000 V), in a cooled horizontal electrophoresis unit (IPGphor, Pharmacia). For the second dimension, the IPG strips were equilibrated for 20 min in 50 mM Tris–HCl, 6% urea, 10% glycerol, 2% SDS, and a trace of bromphenol blue and loaded on a 16% SDS–PAGE gel.

RESULTS

Molecular Typing of Anchorless and Wild Type Prions—Immunoblots with 6D11 of non-PK-treated brain homogenates (BHs) from “anchorless PrP” Tg mice and “anchored PrP” C57BL mice, both infected with the RML scrapie strain, showed that anchorless PrP resolved in 2 bands (Fig. 1A, lane 2), representing mono- and unglycosylated forms, as opposed to the customary separation of anchored PrP under di-, mono- and unglycosylated species (Fig. 1A, lane 3). As expected, the unglycosylated anchorless PrP band co-migrated with the PrP23–230 synthetic peptide at 23 kDa (Fig. 1A, lanes 1 and 2). Higher molecular mass forms, consistent with PrP multimers, in addition to a barely detectable 16 kDa truncated fragment, were observed in “anchorless PrP” Tg mice; conversely, anchored PrP showed an additional C-terminal truncated fragment migrating in a 19-kDa zone, accounting for the C2 fragment, or the unglycosylated fragment generated by endogenous proteolysis (5). Following treatment with PK, anchorless PrPSc separated under mono- and unglycosylated isoforms, migrating at ~21 and 16 kDa (Fig. 1A, lane 4), whereas anchored PrPSc glycoforms migrated at ~28, 24, and 19 kDa (Fig. 1A, lane 5). Hence, the unglycosylated fragment of anchorless PrPSc migrated ~3 kDa faster than the corresponding fragment of anchored PrPSc, in keeping with the estimated molecular mass of the GPI anchor. Enzymatic deglycosylation of anchorless PrP yielded a 23-kDa band, in addition to a barely visible 16 kDa band, and reduced anchored PrP to two distinct bands of 26 and 19 kDa, representing full-length PrP and the C2 fragment (Fig. 1A, lanes 6 and 7); further treatment with PK generated a core fragment of 16 kDa for anchorless PrP and 19 kDa for anchored PrP (Fig. 1A, lanes 8 and 9). Membranes probed with mAb SAF70 overlapped results observed with 6D11 (Fig. 1B, lanes 2–9), with the exception of an additional lower band detected in wild type mice, corresponding to the C1 fragment (Fig. 1B, lane 7). Taken together, the foregoing results were consistent with the GPI anchor contributing for ~3 kDa to the orthogonal migration of PrP, either before or after PK proteolysis.

Two-Dimensional Mapping of Anchorless and Wild Type Prions—By 2D-PAGE, we next compared the isoelectric point (reflecting the net charge) and the mobility (determined by the molecular mass) of anchorless PrP and anchored PrP glycoforms. Reference SDS-PAGE and 2D-PAGE maps of human synthetic PrP peptides were as follows: the 23–230 peptide migrated at 23 kDa, pI 9.4, the 90–230 at 16 kDa, pI 7.9, and the 105–230 at 14.5 kDa, pI 6.6 (Fig. 2A, panels a, b, and table). Immunoblots with 6D11 (data not shown) and SAF70 of BHs from anchorless PrP Tg mice showed that the unglyco-
sylated PrP isoform migrated at 23 kDa, pI 9.4, matching the orthogonal and horizontal mobilities of the synthetic PrP peptide 23–230, whereas monoglycosylated isoforms showed a string pattern in a 28-kDa zone, pI 8–9.2 (Fig. 2B, panels a and b), all reduced to a single 23-kDa spot upon enzymatic deglycosylation (Fig. 2B, panels c and d). In overexposed films a 16-kDa spot, matching the migration of the 90–230 peptide, was seen.

Immunoblots with 6D11 of wt mice BHs showed that PrP separated as trains of spots migrating between 35 and 19 kDa, pIs 4–8.5, including glycosylated and unglycosylated isoforms of the full-length PrP and glycoforms of the C2 fragment (data not shown); upon deglycosylation, PrP isoforms resumed to two main 26-kDa and 19-kDa spots, pIs 8.4 and 7.2–8.2, accounting for the unglycosylated full-length PrP and the C2 fragment. Membranes probed with SAF70, which preferentially...
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binder glycosylated forms of C1, disclosed a PrP pattern dominated by a set of spots with an acidic migration (Fig. 2B, panels e and f), all reduced to three set of spots after deglycosylation, including full-length PrP, C2, and C1 fragments (Fig. 2B, panels g and h). Notably, intra-sample differences in PrP decoration, observed between SDS-PAGE and 2D-PAGE, reflect the facilitation or restriction of mAbs binding to PrP molecules denatured by alternate protocols.

Taken together, using a high sensitive proteomic approach we confirmed that lack of the GPI anchor induces an alkaline shift of ~1 pH unit in the horizontal migration of PrP, and also that anchorless PrP is characterized by immature glycosylation and altered endogenous proteolytic processing (13).

After PK digestion, anchorless PrPSc separated into minor glycosylated spots in a 19-kDa zone, in addition to well-represented unglycosylated 16 kDa isoforms, consistent with multiple ragged N-terminal ends of the unglycosylated PrP27–30 fragment (Fig. 2A, panels a and b). After deglycosylation C-terminal truncated fragments (CTFs), migrating at 14–16 kDa, pl range 4–6, were observed (Fig. 2C, panels g and h). Taken together, besides differences in migration, anchorless PrPSc does not generate CTFs under protease treatment.

**Molecular Characteristics of PrPSc in sCJD Subtypes and vCJD—**In previous 2D-PAGE studies of PrPSc in different sCJD subtypes and vCJD, we were unable to detect molecular forms consistent with anchorless PrP species. Here, we first assessed the SDS-PAGE migration of type-1 and type-2 PrPSc, as compared with the map of human synthetic PrP peptides. Accordingly, the unglycosylated PrP27–30 fragment migrates at 19.5-kDa in type-1 PrPSc, and 17-kDa in type-2 PrPSc (Fig. 3, A and B), which is at variance with their conventionally reported migration at 21 and 19 kDa (20). Next, we reassessed the 2D-PAGE migration of the core fragment in different sCJD subtypes using anti-PrP mAbs 3F4 and 3E2. In sCJD MM1, the PrP27–30 core fragment migrates as a single tailed 19.5-kDa spot centered at pl 7.0, whereas in sCJD MM2 the core fragment separated as three major 17.5-kDa spots, pl range 6.0–7.0, as an effect of multiple cleavage sites (Fig. 3C). Differences in migration between PrPSc of MM1 and MM2 subtypes were better visualized in a sCJD case with co-occurrence of both PrP types (MM1 + 2), where the core fragment of type-1 PrPSc and the most basic spot of the type-2 PrPSc train shared a spot at pl 7.0, with diverging molecular masses. In contrast to sCJD MM1, in MV2 and VV2 subtypes PrPSc separated as multiple spots in a wider pl range, spanning from 6.6 to 8.0, indicating a larger heterogeneity of PrP N-terminal variants, whereas in vCJD, PrPSc resolved in three major spots (18, 21).

By probing membranes with mAb 3E2 (epitope 214–231), the 2D-patterns of PrPSc core fragments in different sCJD subtypes and vCJD overlapped those observed with 3F4; additionally, acidic C-terminal truncated fragments were seen in distinct sCJD subtypes, as already reported (Fig. 3D). Taken together, in MM1, MM2, and MM1 + 2 sCJD subtypes, each single spot of the core fragment is 1.0 pH unit more acidic than the corresponding synthetic peptide and ~2.5 kDa slower, as expected for anchored PrP species. At variance, MV2, VV2, and vCJD show the presence of additional basic spots with migration between 7.0 and 8.0. Although caution is needed in assigning these species to anchorless PrP forms, the lack of the expected 2.5 kDa shift in their orthogonal migration and the recognition by mAb3E2 rule out the hypothesis that they could represent anchorless PrP species, rather suggesting the presence of molecular forms with modified GPI composition.

**Antibody Mapping of PrPSc in sCJD and vCJD: No Evidence for Anchorless Species—**Previously, Notari et al. reported on the constitutive expression of anchorless PrPSc species in all sCJD subtypes, and their enrichment after PK digestion (16). Anchorless PrP quasispecies were tentatively identified based on their migration, ~2 kDa faster than the core fragment, and their detection with mAbs directed to epitopes along PrP residues 89–221, including 12B2 and 3F4, but not with a rabbit antiserum directed to residues 220–231.

In the present study, PrPSc characterization in different sCJD subtypes and vCJD, did not show additional bands, consistent with anchorless PrP species, either before (Fig. 4A, panels a and b) or after PK treatment (Fig. 4A, panels c and d). In particular, immunoblots with anti-PrP mAbs 6D11 (epitope 93–109) and ICSM-35 (epitope 93–102) showed only a different affinity and variability of the PrPSc glycosylation profile, but no additional faster migrating bands (data not shown). At variance, immunoblots with 12B2, recognizing an epitope at position 89–93 of human PrP, showed that the unglycosylated PrP27–30 fragment co-migrated in a 19.5 kDa zone in all sCJD subtypes, either with type-1 or type-2 PrPSc and vCJD. An exception to this pattern was the lack of recognition of PrP27–30 in CJD MM2 subtype, and the detection of a PrP band migrating in a 17 kDa zone in four out seven sCJD MV2 cases (Fig. 4B, panels a and b). These findings further support previous demonstration that type-1 PrPSc is variably expressed in vCJD and sCJD cases with type-2 PrPSc, either homozygous or heterozygous at codon 129. The lack of PrP27–30 staining in MM2 cases is consistent with this molecular subtype being mostly composed by the N-terminal variant of PrP27–30 starting at S97, hence lacking the 12B2 epitope (21).

After 2D-PAGE separation, immunoblots with 12B2 confirmed the co-expression of type-1 and type-2 unglycosylated PrP27–30 spots in four MV2 cases. As expected, these spots shared a similar horizontal migration, within a 6.0–7.0 pl range, but differed in their orthogonal mobility at 19.5 and 17 kDa (Fig. 4C). As compared with the pattern obtained with mAbs 3E2 and 3F4 (see Fig. 3, C and D), in MV2 cases the reduced number of spots, and in particular the absence of basic 17.5-kDa isoforms, is consistent with mAb 12B2 (epitope 89–93) missing the capture of G92, S97, and W99 N-terminal residues 89–221, including 12B2 and 3F4, but not with a rabbit antiserum directed to residues 220–231. Therefore, using an extensive panel of anti-PrP mAbs and a high sensitive separation technique, we were unable to confirm the presence of anchorless PrP species, as suggested by Notari et al. using a conventional SDS-PAGE technique and an anti-PrP rabbit antiserum (16).
FIGURE 3. SDS-PAGE and 2D-PAGE analyses of PrP<sup>Sc</sup> in sCJD and vCJD. A, immunoblot with 6H4 of BH from frontal cortices of sCJD subtypes MM1, MV2, and synthetic PrP peptides, show that the PK-resistant unglycosylated PrP<sup>Sc</sup> fragment is 19.5 kDa for type 1 and 17 kDa for type 2 PrP<sup>Sc</sup>. B, molecular masses and pI of synthetic PrP peptides matching major N-terminal variants of type 1 and type 2 PrP<sup>Sc</sup> detected in human prion diseases. C, 2D-PAGE immunoblots with 3F4 of PrP<sup>Sc</sup> core fragments in different molecular sCJD subtypes. In MM1 sCJD subtype, PrP<sup>Sc</sup> core fragment consists of a single 19.5 kDa spot, pI 7.0, whereas in MM2 it is composed by three 17-kDa spots, pI range 6.0–7.0; distinct migration of PrP<sup>Sc</sup> core fragment is shown in a MM1/2 sCJD case (arrows indicate different PrP<sup>Sc</sup> types. In VV2, MV2, and vCJD, PrP<sup>Sc</sup> separates as multiple 17-kDa spots, pI range 6.6–8.0. D, immunoblots with 3E2 of BHs reported in panel C, show an overlapping pattern of PrP<sup>Sc</sup> core fragments, in addition to additional sets of acidic spots representing CTFs (arrows).
GSS-like Internal PrP\textsuperscript{Sc} Fragments Are Generated in “Anchorless PrP” Tg Mice, but Not in sCJD and vCJD, following PK Digestion under Inactivating Conditions—We next investigated the influence of GPI anchor in PrP\textsuperscript{Sc} conformation under conditions where PMSF was omitted. Using this protocol, PK-digested BHs from anchorless PrP Tg mice, showed, in addition to PrP\textsubscript{27–30}, bands migrating at 14 and 8 kDa, both recognized by mAbs binding to PrP residues 89–109, including 12B2,
6D11, and ICSM-35 (Fig. 5A). Conversely, mAb 6H4 faintly stained the 14-kDa band, and 4G11 detected barely visible bands in a higher molecular zone, corresponding to previously identified C-terminal fragments (Fig. 5A). At variance, the 14 and 8-kDa bands were not observed in anchored PrP C57BL mice. Interestingly, omission of PMSF induced a reduction of large PrP aggregates, migrating in a high zone, suggesting that these aggregates originate the PrP97–30 in wild type mice and internal fragments in “anchorless PrP” mice, either at 1 or 4 h of PK digestion (Fig. 5B). Under the same experimental conditions, no truncated fragments migrating in the 14–8 kDa range were observed in brain homogenates from different sCJD subtypes (Fig. 5C).

FIGURE 5. PK digestion under inactivating conditions generates GSS-like internal PrPSc fragments in “anchorless PrP” Tg mice, but not in sCJD and vCJD. A, immunoblot analysis of wild type (odd numbers) and “anchorless PrP” Tg mice (even numbers) BHs, before (−) and after (+) PK treatment. In “anchorless PrP mice” PK treatment under inactivating conditions, generates two bands migrating at 14- and 8-kDa, recognized by 12B2, 6D11, and ICSM-35 (lanes 4, 8, 12, arrows), but not 4G11; on the contrary, 6H4 decorates a faint 14-kDa band. B, immunoblots with 6D11 of BHs from “anchorless PrP” mice and “anchorless PrP” Tg mice digested with 50 µg/ml PK at different time intervals. At 1 and 4 h, PK digestion, under inactivating conditions, generates PrP fragments of 14- and 8-kDa in “anchorless PrP” Tg mice but not in “anchored PrP” mice (arrows), paralleled by a decrease of high molecular mass PrP aggregates (brackets). C, immunoblots with 3F4 and 6D11 of sCJD MM1 and MV2 BHs digested with 50 µg/ml PK for 1 and 4 h under standard and inactivating conditions, show the absence of internal PrP fragments.  

**FIGURE 4. Antibody mapping of PrPSc in sCJD and vCJD: absence of unanchored PrPSc forms.** A, immunoblots with mAbs 3F4 and 12B2 of native (panels a and b) and PK-treated (panels c and d) BHs from different sCJD subtypes and vCJD. In PK-untreated samples, PrP species separate in a 35-to-19.5-kDa zone in MM1, and 35-to-17 kDa in MM2, the faster band accounting for the C2 fragment. After PK digestion, the conventional pattern of PrP27–30 is shown with 3F4; conversely, in sCJD MV2 subtype, immunoblot with 12B2 shows the customary di- and monoglycosylated bands, in addition to two unglycosylated bands, the slower co-migrating with the unglycosylated fragment of MM1 subtype, and the faster at 17-kDa (arrow). B, immunoblot with 12B2 of PK-treated BH from MM1, MV1, and VV2 sCJD subtypes shows co-migration of PrP27–30 glycoforms (panel a); conversely, the PrP27–30 pattern in MV2 subtype includes 4 bands, the faster migrating in 17 kDa zone (panel b). C, SDS-PAGE and 2D-PAGE immunoblots with 12B2 of PK-treated MM1 BHs (panels a–c) and MV2 sCJD subtypes (panels d–f) shows that in MM1, PrP27–30 separates as three 30-to-19.5-kDa bands (panel a) that resolve into three sets of spots (panel b) accounting for differently glycosylated species, all reduced to a main 19.5-kDa spot, pl 7.0, after deglycosylation (panel c). In MV2 subtype, 12B2 decorates three major 30-to-19.5-kDa bands, in addition to a 17-kDa band (panel d, arrow), that separates into four sets of spots; following PNGase F treatment, two train of spots at 19.5 and 17-kDa are seen, accounting for type-1 and type-2 core fragments (panel f, arrows). D, schematic representation of the core fragment patterns obtained with 3F4 and 12B2 in MM1 and MV2 sCJD subtypes, combining results shown in Figs. 3C and 4C.
In human prion disorders, the detection of N- and C-terminally PrPSc truncated fragments is a peculiarity of GSS, and, therefore, we compared the electrophoretic migration of PrP fragments detected in “anchorless PrP” Tg mice with brain samples from PrP-CAA Y145Stop and GSS F198S mutations. Under inactivating conditions, immunoblots with 12B2 of PK-digested BHs from Y145Stop, showed the presence of major bands, accounting for the monomeric 8-kDa fragment and multimeric forms thereof, whereas in F198S an additional band of 11 kDa was observed (Fig. 6A, panel a). Intriguingly, the 8-kDa band co-migrated with the faster anchorless PrP internal fragment detected in “anchorless PrP” Tg mice as well as with the synthetic peptide spanning PrP sequence 82–146. As expected, 4G11 failed to recognize internal PrP fragments (Fig. 6A, panel b). Taken together, generation of the internal PrP fragment is a feature of anchorless prions, but not of anchored prions.

Additional evidence that the 8-kDa PrP fragment detected in “anchorless PrP” Tg mice shared its electrophoretic properties with the 82–146 synthetic peptide and with the internal fragment generated by PK digestion of Y145Stop and F189S mutations, was provided by results obtained following 2D-PAGE.
separation (Fig. 6B, panels a–f). However, whereas the 8-kDa fragment migrated as a single spot in "anchorless PrP" Tg mice, in Y145Stop and F198S mutations the internal PK-resistant fragments separated as multiple spots, within an 8.4–9.6 pl zone, as an effect of ragged N and C termini.

**DISCUSSION**

Over the last few years, the identification and characterization of GPI-anchored and GPI-anchorless forms of PrPSc in human and animal prion diseases has been a matter of extensive investigations, aimed at addressing how the diverging physical properties of prions may influence propagation, pathogenesis, and neuropathology of prion disorders. In *in vitro* studies we have previously shown that anchorless PrP undergoes an aberrant metabolism, which includes defective glycosylation, altered transport to the secretory pathway, and lack of recycling through to the plasma membrane (13, 14). These features are in keeping with the present evidence that endogenous proteolysis of brain tissues from RML-infected "anchorless PrP" Tg mice generate minimal amounts of the C2 fragment (mostly derived from PrPSc) and almost undetectable quantities of the C1 fragment (generated by α-cleavage of PrPSc). This is at variance with "anchored PrP" C57BL mice infected with RML that showed well-detectable levels of both fragments. In addition to different levels of PrP species recovered in untreated brain homogenates, under exogenous proteolytic treatment, "anchorless PrP" Tg mice lacked C-terminal truncated fragments, indicating that their generation occurs as a result of an aberrant cleavage of anchored PrPSc. Further differences between "anchorless PrP" Tg mice and "anchored PrP" C57BL mice were observed after PK digestion under inactivating conditions, due to the pruning of anchorless prions, but not anchored prions, at the N and C termini, as a likely effect of conformational changes exposing the anchorless C terminus to proteolytic cleavage. Although the relative amount of internal PrPSc fragments was small, these results suggest that PrP species lacking the amino and carboxyl termini represent a molecular fingerprint of anchorless prions. Intriguingly, a 10-kDa internal fragment has been detected in brain tissues of uninfected Tg mice overexpressing anchorless PrP, and in Tg mice co-expressing anchored and anchorless PrP (12). Noteworthy, the aforementioned transgenic mice develop a spontaneous neurologic illness characterized by large PrP amyloid deposits composed by a 10-kDa PK-resistant fragment. Among human prion diseases, anchorless C-terminally truncated PrPSc fragments are a feature of PrP-CAA and GSS stop codon mutations Y145X, Q160X, Y163X, Y226X, and Q227X (22–25). In addition, in a number of GSS cases associated with point mutations, such as F198S, impaired GPI anchoring has been suggested to cause C-terminal truncation of PrPSc (12). PrP-CAA and/or parenchymal amyloid PrPSc deposition, generating a 7–11 kDa PK-resistant PrP internal fragment, characterize the above mutations at neuropathology, in the absence of spongiosis. Taken together, experimental and human conditions characterized by lack of the GPI anchor or altered GPI anchoring, share common pathogenic mechanisms leading to accumulation of an internal PrP fragment under amyloid plaques.

Recently, Notari *et al.* reported on the presence of anchorless PrPSc in different sCJD subtypes, hence widening the spectrum of molecular PrP quasispecies detected in this human disorder (16). Anchorless PrP forms were tentatively identified based on the immunodecoration of PK-resistant species migrating 2 kDa faster than the unglycosylated PrP27–30, under SDS-PAGE separation. These findings are potentially relevant and raise concern on the potential spreading of anchorless PrP species to body fluids and peripheral tissues of sCJD patients, in view of the propensity of anchorless prions to accumulate in non-neural tissues.

The contribution of the anchor to the molecular mass of PrP has been previously calculated to account for 2–3 kDa and 4 kDa, under experimental conditions using, respectively, hydrofluoric acid and proaerolysin (16, 26). These data are in keeping with our findings showing a 2.5 kDa difference between "anchored PrP" C57BL and "anchorless PrP" Tg mice in the orthogonal migration of either native or PK-resistant PrPSc isoforms. Moreover, our 2D-PAGE immunoblot study discloses a basic shift of 1 pH unit for anchorless species, hence providing an additional parameter for separating anchored and anchorless prions.

Having determined the molecular coordinates of anchorless PrP in Tg mice we focused on the identification of possible anchorless PrPSc forms in sCJD, taking also advantages of a panel of synthetic PrP peptides, as indicators of migration under two-dimensional separation. Based on previous studies, major PK-cleavage sites of PrPSc occur at G78–G82 for type-1 PrPSc, or ~10 amino acids upstream the synthetic 90–230 PrP peptide migrating at ~16 kDa, and G92–S97 for type-2 PrPSc, a few amino acids downstream the above peptide (19). Therefore, the observed migration of the PrP27–30 unglycosylated fragment at 19.5 kDa for type-1 PrPSc and 17 for type-2 PrPSc, is in keeping with their expected molecular masses, taken also in due account the 2.5 kDa contribution of the GPI-anchor moieties. Moreover, the horizontal migration of the synthetic 90–230 PrP milestone at pl 7.9, provided an additional watershed for the detection of anchorless PrP species, knowing that the GPI anchor cause an acidic shift of 1 pH unit. Accordingly, the migration of the core fragment at pl 7.0 in sCJD cases with type-1 PrPSc, and in a 6.0–7.0 pl range in MM2 sCJD, rule out the presence of anchorless PrP species in these sCJD subtypes. At variance, in MV2 and VV2 sCJD and in vCJD, the detection of a few spots with a focusing mobility toward basic pl zones, might apparently suggest the presence of anchorless PrP species. However, basic spots did not show changes in their molecular masses, as compared with acidic spots, therefore suggesting that these isoforms may represent molecular PrP species with modified anchor composition. The latter hypothesis is further supported by the immunodetection of basic spots with mAb 3E2 in a pattern similar to mAb 3F4. These results rule out the possibility of PrPSc truncation at the C terminus, which is expected to compromise antibody recognition. Within this context, the finding of Notari *et al.* that putative anchorless forms were virtually undetectable by 2301 antiserum is not supportive for their presence, being instead a further proof that the extreme C-terminal sequence of PrP molecule is less accessible to mAb recognition following SDS-PAGE separation, unless
exposed to highly denaturing conditions (27, 28). This is also the case of our diverging results obtained with mAb 3E2, which efficiently recognized PrP following 2D-PAGE separation, while leaving PrP unstained in SDS-PAGE immunoblots, thus replicating previous results with mAb SP-214 (18).

Overall, our present data do not support recent findings purporting to show the presence of pools of anchored and anchorless PrP\(^\text{Sc}\) in sCJD with type-1 and type-2A PrP\(^\text{Sc}\). Regarding the detection of anchorless prions in MV2 sCJD subtype, immunoblots with mAb 12B2, which has a high affinity for type-1 PrP\(^\text{Sc}\), clearly showed that the two-dimensional separation of type-1 isoforms at 19.5 kDa and type-2 PrP\(^\text{Sc}\) spots at 17 kDa within a 6.0–7.0 pl range, were consistent with an electrophoretic migration highly suggestive for anchored PrP species. These data are in accordance with recent mass-spectrometry-based analysis of anchorless PrP, showing that PK treatment of anchorless PrP\(^\text{Sc}\) generates several N-terminal ragged ends, leaving unaffected the C terminus (29). Finally, the lack of generating scrapie-infected “anchorless PrP” transgenic and wild type providing scrapie-infected “anchorless PrP” transgenic and wild type mice, and for critical reading of the manuscript.

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