Selective Amplification of Classical and Atypical Prions Using Modified Protein Misfolding Cyclic Amplification*

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Background: The mechanisms of prion strain mutation and its dependence on the environment are not known.

Results: Glycosylation status of PrP⁰ substrate and cofactors controls the selectivity of amplification of classical and atypical PrPSc.

Conclusion: Amplification selectivity of alternative prion states can be regulated by modification of a substrate and cofactors in PMCA.

Significance: Alternative prion states can be selectively amplified from a mixture.

With the development of protein misfolding cyclic amplification (PMCA), the topic of faithful propagation of prion strain-specific structures has been constantly debated. Here we show that by subjecting brain material of a synthetic strain consisting of a mixture of self-replicating states to PMCA, selective amplification of PrPSc could be achieved, and that PMCA mimicked the evolutionary trend observed during serial transmission in animals. On the other hand, using modified PMCA conditions that employ partially deglycosylated PrP⁰ (dgPMCAb), an alternative transmissible state referred to as atypical protease-resistant form of the prion protein (atypical PrPres) was selectively amplified from a mixture. Surprisingly, when hamster-adapted strains (263K and Hyper) were subjected to dgPMCAb, their proteinase K digestion profile underwent a dramatic transformation, suggesting that a mixture of atypical PrPres and PrPSc might be present in brain-derived materials. However, detailed analysis revealed that the proteinase K-resistant profile of PrPSc changed in response to dgPMCAb. Despite these changes, the 263K strain-specific disease phenotype was preserved after passage through dgPMCAb. This study revealed that the change in PrPSc biochemical phenotype does not always represent an irreversible transformation of a strain, but rather demonstrated the existence of a wide range of variation for strain-specific physical features in response to a change in prion replication environment. The current work introduced a new PMCA technique for amplification of atypical PrPres and raised a number of questions about the need for a clever distinction between actual strain mutation and variation of strain-specific features in response to a change in the replication environment.

Prion diseases, or transmissible spongiform encephalopathies, are fatal neurodegenerative disorders that can be sporadic, inherited, or infectious in origin (1). Although the infectious agent in prion diseases lacks informative nucleic acids and, instead, consists of a prion protein (PrP)² in its abnormal, β-sheet rich, self-replicating conformation (PrPSc), Darwinian evolutionary theory concepts including selection and adaptation have been employed to describe vast variations in prion disease phenotype as well as their transformation and the emergence of new ones (2, 3). It has been proposed that prion strains exist as a mixture of quasispecies; the populations of quasispecies are dynamic and can adapt quickly in response to changes in their replication environment (2, 4). Changes in replication environment can be associated with prion transmission from animals to cultured cell lines and/or PMCA reactions and vice versa and associated with supplementing physiologically active compounds or drugs to cultured cells or PMCA reactions (2, 5–7).

Strain-specific differences in the clinical manifestation of the disease are reflected and, to some extent, defined by the biochemical characteristics of PrPSc (8–13). Transformations or mutations of prion strains are often mirrored by changes in PrPSc biochemical phenotype, which includes strain-specific ratio of glycoforms, pattern of PK-resistant products, size of PK-resistant core, PrPSc conformational stability, and so forth (14, 15).

With the development of PMCA techniques, the question of faithful propagation of prion structures is constantly discussed (16–23). On the one hand, PMCA-derived PrPSc products were shown to produce the same strain-specific disease phenotype in animals as brain-derived PrPSc (16, 18, 24). On the other hand, differences in incubation times to disease by brain- and PMCA-derived PrPSc suggest the possibility of change in structure and/or composition of PrPSc populations in response to replication in vitro (23). To what extent can strains be changed in PMCA? Can PMCA select different transmissible states of PrP from a mixture? Can PMCA speed up strain evolution? Does prion adaptation to PMCA represent a reversible change with...

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The abbreviations used are: PrP, prion protein; PrP⁰, cellular isoform of PrP; PrPres, protease-resistant form of the prion protein; PMCA, protein misfolding cyclic amplification; sPMCAb, serial PMCA with beads; PK, proteinase K; PNGase F, N-glycosidase F; BH, brain homogenate; NBH, normal brain homogenate; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; dg, deglycosylated; LMW, low molecular weight.
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out affecting molecular features that account for strain-ness? These questions are addressed in the current study. Here we show that PMCA with beads (PMCAb) (24, 25) can be a valuable method to study the evolutionary potential of prion strains. We showed that by subjecting brain material of a prion strain of synthetic origin that consists of a mixture of self-replicating states to serial PMCAb, selective amplification of pathogenic PrPSc could be achieved, and that serial PMCAb mimicked strain evolution in animals. Using modified PMCAb conditions that employed partially deglycosylated PrP sub-

mimicked strain evolution in animals. Using modified PMCAb conditions that employed partially deglycosylated PrPSc substrate (dgPMCAb), an alternative transmissible PrP state referred to as atypical PrPres was selectively amplified from a mixture. Coupling of PMCAb with dgPMCAb offers a new in vitro approach for elucidating adaptation and selection of transmissible PrP states. Furthermore, the newly introduced dgPMCAb might also be useful for strain typing.

EXPERIMENTAL PROCEDURES

Ethics Statement—This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore (Assurance Number A3200-01; Permit Number 0312020).

Scrapie Brain Material—Synthetic strains SSLOW, LOTSS, and SO5 were produced in golden Syrian hamster upon inoculation of recombinant PrP fibrils as described previously (26–28). 263K and HY brain materials were kindly provided by Robert Rohwer and Richard Bessen, respectively.

Bioassay—We thank Robert Rohwer for assistance in conducting bioassays. Weanling golden Syrian hamsters (all males) were inoculated intracerebrally under 2% O2/4 minimum alveolar concentration isoflurane anesthesia. Each hamster received 50 μl of 10% BH inoculum or PMCAb/dgPMCAb reaction products diluted 10-fold in PBS. After inoculation, hamsters were observed daily for disease using a “blind” scoring protocol. Hamsters without any signs of clinical disease were euthanized at 661 days after inoculation.

Protease K Digestion—Brains were collected aseptically, used to prepare 10% BHs in PBS as described elsewhere (Makarava et al. 26), and digested with 20 μg/ml PK in the presence of 2% sarkosyl in PBS and 50 mm Tris, pH 7.5, for 30 min at 37 °C with 1000 rpm shaking using a Delfia plate shaker (Wallac). PK digestion was stopped by adding SDS sample buffer and heating the samples for 10 min in a boiling water bath. After loading onto NuPAGE 12% Bis-Tris gels and transfer to PVDF membrane, PrP was detected with 3F4 (epitope 109–112) or SAF-84 (epitope 160–170) antibody, as indicated. Protein Misfolding Cyclic Amplification with Beads—10% normal brain homogenate (NBH) from healthy hamsters was prepared as described before (Makarava et al. 27) and used as a substrate for PMCAb (Gonzalez- Montalban et al. 12). For the first round, 10 μl of 10% BH from inoculated animals were added to 90 μl of NBH. The sonication program consisted of 30 s sonication pulses delivered at 50% power efficiency applied every 30 min during a 24-h period. For each subsequent round, 10 μl of the reaction from the previous round were added to 90 μl of fresh substrate. Each PMCAb reaction was carried out in the presence of three 0.094-inch Teflon beads (SmallParts). To minimize the possibility of cross-contamination, the horn chamber was decontaminated after each experiment using 2 N NaOH.

To analyze production of PK-resistant PrP material in PMCAb, 10 μl of sample were supplemented with 5 μl of SDS and 5 μl of PK, to a final concentration of 0.25% SDS and 50 μg/ml PK, unless noted otherwise, followed by incubation at 37 °C for 1 h. The digestion was terminated by the addition of SDS-sample buffer and heating the samples for 10 min in a boiling water bath. Samples were loaded onto NuPAGE 12% Bis-Tris gels, transferred to PVDF membrane, and probed with 3F4 or SAF-84 antibodies.

Protein Misfolding Cyclic Amplification with Partially Deglycosylated Substrate (dgPMCAb)—To produce partially deglycosylated substrate, 10% NBH from healthy hamsters prepared for PMCAb (see above) was treated with PNGase F (New England Biolabs, glycerol-free) as follows. After preclearance of NBH at 500 x g for 2 min, 1500 units/ml PNGase F were added to the supernatant, and the reaction was incubated on a rotator at 37 °C for 5 h. The resulting substrate was used in dgPMCAb using the sonication protocol described for PMCAb. To prepare RNA-depleted dgPMCA substrate, 100 μg/ml RNase A (Sigma, catalog number R4875) was added to the NBH following PNGase F treatment, and the reaction was incubated on a rotator at 37 °C for 1 h as described previously (Gonzalez-Montalban et al. 19).

Deglycosylation of PrPres—Removal of N-linked glycans was performed using glycerol-free PNGase F and supplied buffers (New England Biolabs). 10% BH or dgPMCAb products were mixed with equal volume of 4% sarkosyl in PBS, pH 7.4, and digested with 20 or 500 μg/ml PK as indicated, deglycosylated following the procedure described elsewhere (Makarava et al. 26), and assayed by Western blot with SAF-84 antibody.

RESULTS

Standard PMCAb Amplifies PrPSc, whereas dgPMCAb Preferentially Amplifies Atypical PrPres—To elucidate amplification requirements for PrPSc and atypical PrPres, brain materials from animals of the first or second passage of the synthetic strain LOTSS containing mixtures of atypical PrPres and PrPSc were used (Fig. 1A). Although PrPSc can be detected by both 3F4 (epitope 109–112) and SAF-84 (epitope 160–170) antibodies, atypical PrPres reacts only with C-terminal antibody SAF-84 (Fig. 1A) (27, 28). PK treatment of atypical PrPres produces three bands of ~23, 16, and 13 kDa, which correspond to di-, mono-, and unglycosylated glycoforms of PK-resistant fragment encompassing residues ~152/153–231 (Fig. 1A). The brain materials from LOTSS first passage predominantly contained atypical PrPres, whereas LOTSS second passage showed a mixture of atypical and PrPSc (Fig. 1A).

To prepare a substrate for dgPMCAb, hamster NBH was treated with PNGase F under nondenaturing conditions. Such treatment shifted the ratio of the three PrPSc glycoforms from predominantly diglycosylated to predominantly monoglycosylated forms (Fig. 1B). When brain material that contained a mixture of PrPSc and atypical PrPres was used for seeding PMCAb reactions, PMCAb selectively amplified PrPSc (Fig.
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The PK-resistant core of brain-derived atypical PrPres consists of the C-terminal region ~153–231 (Fig. 1D) (28). If PK digestions of the dgPMCAb-derived atypical PrPres were performed with high concentrations of PK (>100 μg/ml), the PK digestion pattern of dgPMCA-derived atypical PrPres resembled that of the brain-derived seeds (Figs. 1D and 2A, A and B). However, at standard PK concentrations (20–100 μg/ml), the band that corresponded to unglycosylated dgPMCAb-derived atypical PrPres appeared as a doublet at 13 and 14 kDa (Fig. 2A, A and B). The 14-kDa band was produced as a result of PK cleavage at a site N-terminal to the residue 153. Absence of a doublet in the brain-derived material can be explained by the fact that atypical PrPres was exposed to proteases during the lifespan of inoculated animals and thus accumulated in its most proteolytically digested form (Fig. 2B). In fact, even in the absence of PK digestion, brain-derived atypical PrPres appeared in gels as mostly cleaved (Figs. 1C and 2B).

Similar to brain-derived atypical PrPres, three bands of dgPMCA-derived products represent three glycoforms. However, because dgPMCA-derived products were formed using partially deglycosylated PrPSc substrate, the ratios of three glycoforms in dgPMCA- and brain-derived atypical PrPres were slightly different (Fig. 1D). Consistent with previous data (28), amplification of PrPSc was RNA-dependent, whereas amplification of atypical PrPres was RNA-independent (Fig. 1E). The effect of RNA on amplification rate was analyzed using the experimental format, where reactions were seeded with incrementing dilutions of PrPSc propagated in PMCAb or atypical PrPres propagated in dgPMCAb and conducted in RNA-depleted BH or NBH. Indeed, in RNA-depleted BH, the amplification rate of PrPSc dropped by 10-fold when compared with NBH, whereas the amplification rate of atypical PrPres was not affected by RNA depletion (Fig. 1E). In summary, these results demonstrated that PrPSc and atypical PrPres exhibited different molecular features and could be selectively amplified under two PMCAb conditions that employ unmodified or partially deglycosylated PrPSc as a substrate.

How Selective Is the Amplification of PrPSc or Atypical PrPres in PMCAb and dgPMCAb, Respectively?—Next we were interested in testing whether PMCAb and dgPMCAb could be employed for generating material free of atypical PrPres or PrPSc. To test whether a mixture of atypical PrPres and PrPSc could be completely cleared of atypical PrPres, sPMCAb reactions were seeded with LOTSS brain materials from the first or second passage, which both contain large amounts of atypical PrPres and smaller amounts of PrPSc, and subjected to four serial rounds. Then, serial dgPMCAb was used for testing whether residual amounts of atypical PrPres remained (Fig. 3A). After only four PMCAb rounds, no atypical PrPres could be detected in PMCAb-derived materials by serial dgPMCAb in experiments seeded with LOTSS first or second passage materials (Fig. 3B). Notably, although dgPMCAb preferentially amplified atypical PrPres (Fig. 1), PrPSc could be amplified in dgPMCAb with a reasonably high rate, too, if seeding material lacked atypical PrPres that competes for a substrate. This experiment indicated that brain material containing excessive

**FIGURE 1. Analysis of amplification requirements for PrPSc and atypical PrPres.** A, analysis of PK-resistant material in animals from the LOTSS first, second, or third passage. 5% LOTSS first passage and 1% LOTSS second and third passage BH were used. Western blots were stained with 3F4 (left panel) or SAF-84 (right panel) antibodies. passag. #, passage number. B, analysis of PrPSc glycoform composition in NBH- or PNGase F-treated NBH using 3F4 (left panel) or SAF-84 (right panel) antibodies. SAF-84 detects PrPSc and naturally occurring products of PrPSc cleavage (C1). C, serial PMCAb or dgPMCAb reactions were seeded with 102-fold diluted brain material from LOTSS second passage. Reaction products were treated with 500 μg/ml PK, and Western blots were stained with SAF-84 (top panel) or 3F4 (bottom panel) antibodies. D, analysis of dgPMCAb-derived and brain-derived (LOTSS first passage) atypical PrPres PK-resistant products prior to and after PNGase F treatment. dgPMCAb- and brain-derived materials were treated with 500 or 20 μg/ml PK, respectively. Western blot was stained with SAF-84 antibody. In panels A, B, and D, diglycosylated, monoglycosylated and unglycosylated glycoforms are marked as 2, 1, and 0, respectively. E, analysis of RNA dependence of amplification of PrPSc (top panel) or atypical PrPres (bottom panel). Reaction products of the fourth round of PMCAb or dgPMCAb seeded with LOTSS second passage were diluted 10-, 30-, 100-, 300-, or 1000-fold, as indicated, into 10% NBH or RNA-depleted NBH and amplified for one PMCAb (top panel) or dgPMCAb (bottom panel) round, respectively. Dilution of seeds without amplification is shown as a control. Reaction products were treated with 50 μg/ml PK. Western blots were stained with SAF-84 antibody.

**FIGURE 2. Comparison of PK resistance profiles for dgPMCAb-derived and brain-derived atypical PrPres.** A and B, dgPMCAb-derived (A) or brain-derived (B) atypical PrPres materials from LOTSS first passage were treated with increasing concentrations of glycerol-free proteinase K (Sigma catalog number P66556) in the presence of 0.25% SDS for 1 h at 37 °C. To produce dgPMCAb-derived material, brain material from LOTSS first passage was subjected to four serial dgPMCAb rounds. Western blots were stained with SAF-84 antibody.
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To test whether atypical PrPres material free from PrPSc could be produced, serial dgPMCAb reactions were seeded with LOTSS brain material from the second passage, subjected to four serial rounds in NBH and for seven additional rounds in RNA-depleted BH. Then, serial PMCAb was used to test whether residual amounts of PrPSc remained in dgPMCAb-derived material (Fig. 3, C and D). Indeed, PMCAb revealed the presence of PrPSc, which was detected starting from the second sPMCAb round (Fig. 3D). This result indicated that dgPMCAb amplified PrPSc in parallel with atypical PrPres, although at very low levels. This result was also consistent with the hypothesis that atypical PrPres can give rise to PrPSc in PMCAb (28). Currently, it is difficult to distinguish between two possibilities.

PMCAb Mimicked the Process of Strain Evolution in Animals—Previously, we found that three serial passages of LOTSS starting from inoculation of recombinant PrP fibrils, which is referred to as first passage, were required for developing clinical signs of prion disease (27). Notably, the animals from the first or second passages accumulated large amounts of atypical PrPres (Fig. 1A), and second passage animals showed substantial amounts of PrPSc (Fig. 1A), whereas the clinical disease was observed only starting from the third LOTSS passage. We were interested in testing whether PMCAb could speed up the natural course of synthetic strain evolution by selectively amplifying a pathogenic component from the brain material of the LOTSS first passage.

PMCAb reactions were seeded with brain material from the LOTSS first passage that contained predominantly atypical PrPres (Fig. 1A), subjected to eight serial rounds, and infectivity of PMCAb products was tested in animals. In contrast to the animals inoculated with the seeds (brain material from the LOTSS first passage), the animals that received sPMCAb products developed clinical symptoms with a 100% attack rate (Table 1). The clinical manifestation of the disease produced by sPMCAb products was indistinguishable from that observed for the LOTSS third passage. Moreover, animals inoculated with PMCAb products showed only PrPSc in their brain material, whereas brain material from the LOTSS second and even third passages contained atypical PrPres (Fig. 4A). Together with the previous results, this experiment illustrated that passaging of brain material with predominantly atypical PrPres through PMCAb selectively amplifies PrPSc, which is pathogenic to animals.

It is noteworthy that the incubation time to disease was longer in animals inoculated with the sPMCAb products than in LOTSS third passage animals (Table 1). Longer incubation periods for PMCA/PMCAb products than for brain-derived material were previously observed for other strains including 263K (23, 24). In fact, in a series of control experiments, animals inoculated with the products of PMCAb reactions seeded with the brain material from SSLOW first passage also displayed a longer time to the disease than animals from SSLOW second or third passages (Table 1). Taking into account that SSLOW brain material did not have any detectable amounts of atypical PrPres (Fig. 4B), a longer incubation time for PMCA/PMCAb-derived versus brain derived material could be explained by a PMCA/PMCAb-induced reversible change in the PrPSc population, which is well adapted to replicate quickly in PMCA/PMCAb but not in animal brains (19).

It is important to note that although dgPMCAb preferentially amplified atypical PrPres, PrPSc also amplified in dgPMCAb, although at lower levels (Fig. 3). PrPSc amplification at very low levels occurred even in dgPMCAb carried out using RNA-depleted substrate. Although PrPSc remained undetected in dgPMCAb products by Western blot, dgPMCAb products readily seed PrPSc in standard PMCAb. For these reasons, it is currently not possible to produce atypical PrPres free of residual amounts of PrPSc in dgPMCAb and test its infectivity in animal studies.

263K and HY Produce Atypical Pattern of PK-resistant Products When Amplified in dgPMCAb—Having found that dgPMCAb was able to selectively amplify atypical PrPres from strains of synthetic origin including LOTSS and S05 (27, 28), we were interested in testing whether dgPMCAb could detect alternative self-propagating states in common rodent strains such as 263K or Hyper (HY). Indeed, when seeded with 263K or HY brain materials, dgPMCAb reactions produced atypical PK resistance patterns that consisted of low molecular weight (LMW) products in addition to PrP27–30, a standard PK-resistant core of PrPSc (Fig. 5, A and B). However, in contrast to atypical PrPres of LOTSS or S05, the LMW products of 263K and HY did not replace but rather accompanied PrP27–30 in serial dgPMCAb.

SSLOW, a synthetic strain that lacked atypical PrPres in brain material as evident from Western blotting (26), also produced LMW products in dgPMCAb in addition to PrP27–30 (Fig. 5C). Interestingly, a consistent change in mobility of SSLOW LMW products was detected in serial dgPMCAb, a phenomenon observed in several independent experiments (Fig. 5D). This shift indicated that propagation of SSLOW seeds...
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TABLE 1
Incubation time to disease for LOTSS and SSLOW brain material subjected to sPMCAb

| Strain, passage number | Inoculum | Incubation time (days), mean ± S.D. | Attack rate |
|------------------------|----------|-----------------------------------|-------------|
| LOTSS, second passage   | 10% BH sPMCAb products<sup>a</sup> | 544 ± 20 | 8/8 |
| LOTSS, second passage after sPMCAb | sPMCAb products<sup>a</sup> | 356 ± 27 | 8/8 |
| LOTSS, third passage    | 10% BH sPMCAb products<sup>a</sup> | 481 ± 4 | 8/8 |
| SSLOW, second passage   | 10% BH sPMCAb products<sup>a</sup> | 503 ± 21 | 7/7 |
| SSLOW, third passage    | 10% BH sPMCAb products<sup>a</sup> | 318 ± 16 | 23/23 |

<sup>a</sup> No clinical signs for as late as 660 days after inoculation.

<sup>b</sup> sPMCAb products were diluted 10 fold prior to inoculation.

What Is the Origin of LMW Bands in dgPMCAb?

To test the above hypothesis, we first employed PMCAb conditions that selectively amplify atypical PrPres. Although amplification of PrP<sup>Sc</sup> is known to be RNA-dependent, the amplification of atypical PrPres does not depend on RNA (Fig. 1E) (28). To validate this approach, atypical PrPres of LOTSS or S05 was shown to amplify readily in dgPMCAb in the absence of RNA (Fig. 6A). In contrast, the amplification rates of 263K, HY, or SSLOW LMW products in dgPMCAb with RNA-depleted BH dropped in parallel with the amplification rates of the corresponding PrP<sup>Sc</sup> (Fig. 6A). In fact, no PK-resistant signal was detected after the second dgPMCAb round for 263K, HY, or SSLOW in RNA-depleted BH. Furthermore, in the alternative experimental format introduced in Fig. 1E, atypical PrPres of S05 could be amplified with the same rates regardless of the presence or absence of RNA (Fig. 6D). In contrast, in the absence of RNA, very weak if any amplification of 263K or HY LMW products was observed (Fig. 6, B and C). In fact, LMW products disappeared in parallel with PrP<sup>Sc</sup> upon an increase in seed dilution factor. In summary, regardless of the experimental format or PMCAb conditions used, we were not able to dissociate 263K, HY, or SSLOW LMW products from the corresponding PrP<sup>Sc</sup>. These results support the second hypothesis that the LMW products were produced as a result of PrP<sup>Sc</sup> cleavage at internal sites.

An alternative approach for testing the origin of the LMW products involved PMCAb conditions that selectively amplified PrP<sup>Sc</sup> and testing whether LMW products remained in PMCAb-derived material that was cleared of atypical PrPres. To validate this approach, LOTSS brain materials containing mixtures of atypical PrPres and PrP<sup>Sc</sup> were subjected to four

in vitro was accompanied by some minor, but consistent change in their biochemical features.

What Is the Origin of LMW Bands in dgPMCAb?—Two alternative hypotheses could be put forward to explain the origin of 263K, HY, or SSLOW LMW products in dgPMCAb. It is tempting to speculate that minor amounts of atypical PrPres are always present in 263K, HY, or SSLOW brain materials. Alternatively, the LMW products could be generated due to cleavage of classical PrP<sup>Sc</sup> at internal sites. The second hypothesis postulates that when exposed to the dgPMCAb environment, PrP<sup>Sc</sup> adapts to the amplification conditions that utilize partially deglycosylated PrP<sup>Sc</sup> as a substrate. As a result, internal sites in dgPMCAb-derived PrP<sup>Sc</sup> become more accessible to proteinase K than in brain- or PMCAb-derived PrP<sup>Sc</sup>.

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FIGURE 6. Elucidating the origin of 263K, HY, and SSLOW LMW PK-resistant products. A, analysis of PK-resistant products produced in serial dgPMCAb reactions conducted in RNA-depleted BH (RNA-depl.BH) and seeded with 10²-fold diluted 263K, HY, SSLOW second passage, LOTSS second passage, or S05 second passage brain material. B–D, analysis of amplification rates of PK-resistant materials in dgPMCAb reactions conducted using NBH or RNA-depleted BH as substrates and seeded with dgPMCAb products of 263K (B), HY (C), or S05 second passage (D). 263K, HY or S05 dgPMCAb products were diluted 10-, 30-, 100-, 300-, or 1000-fold, as indicated. Dilution of seeds without amplification is shown as a control. E, 263K or HY brain materials were first subjected to four sPMCAb rounds (not shown) and then to four rounds of dgPMCAb. In all experiments, reaction products were treated with 50 μg/ml PK. Western blots were stained with SAF-84 antibody.

sPMCAb rounds as presented in Fig. 3A. No atypical PrPres could be detected any longer in sPMCAb-derived LOTSS by dgPMCAb (Fig. 3B). In a similar manner, 263K or HY brain materials were subjected to serial PMCAb and then used to seed serial dgPMCAb. The PK resistance pattern with LMW PK-resistant products emerged at the first dgPMCAb round for both strains (Fig. 6E). Again, this result supported the hypothesis that LMW products were generated via cleavage of dgPMCAb-derived PrPSc at internal sites. Assuming that the internal sites were accessible to PK in dgPMCAb-derived PrPSc, we were interested in testing whether brain-derived PrPSc can also produce LMW products if it is treated with large concentrations of PK. Indeed, digestion of brain-derived 263K with high concentrations of PK generated C-terminal LMW products, albeit of very minor intensity (Fig. 7). In contrast to the atypical PrPres from LOTSS, which was observed at standard PK concentrations, 263K brain-derived LMW bands appeared gradually as a function of an increase in PK concentration and, therefore, seemed to be produced via cleavage of PrPSc at internal sites (Fig. 7).

Application of dgPMCAb for Strain Typing—Regardless of whether the dgPMCAb-derived PK digestion profile consists of LMW products that originate from PrPSc or independent self-replicating states such as atypical PrPres, dgPMCAb could be a valuable technique for strain typing. For four of the strains tested here, the PK digestion pattern of dgPMCAb-derived material was unique and more informative than the patterns of brain- or PMCAb-derived material (Fig. 8). In fact, the PK-digested pattern produced from dgPMCAb-derived material can be referred to as a strain-specific "bar code." Interpretation of such a bar code should be considered with great caution as it might reflect individual strain-specific structures of PrPSc and/or the presence of alternative self-replicating states in a mixture with PrPSc.

A Passage of PrPSc through dgPMCAb Does Not Change Strain-specific Features—The previous results established that dgPMCAb could be a valuable tool for amplification of atypical PrPres structures that were not amplifiable in PMCAb. Furthermore, we showed that the PK digestion pattern of dgPMCAb-derived PrPSc was different from that of brain- or PMCAb-derived PrPSc. Although these differences appear to be attributed to the higher accessibility of dgPMCAb-derived PrPSc internal sites to PK cleavage, we cannot exclude that partial deglycosylation of a substrate caused an irreversible change in PrPSc structure and strain mutation. Such a possibility is valid considering recent statements that inhibition of complex glycosylation by swainsonine led to strain mutations and selection of new substrains in cultured cells (2). To test this possibility, brain-derived 263K was propagated in dgPMCAb for eight serial rounds, and then dgPMCAb products were used to seed standard PMCAb and subjected to 10 serial rounds, and PMCAb products were tested in bioassays (Fig. 9A). Already within the first round of PMCAb, the PK digestion pattern of dgPMCAb-derived PrPSc reverted to that typical for brain- or PMCAb-derived 263K (Fig. 9B). This result supports the previous conclusion that the dgPMCAb-specific PK digestion pattern was due to high accessibility of internal sites to PK digestion in dgPMCAb-derived PrPSc. It also illustrates that the ability of dgPMCAb-derived PrPSc to propagate in PMCAb was not diminished. Bioassay experiments revealed that the products obtained after 263K propagation in serial dgPMCAb and then in sPMCAb were identical to those obtained after 263K propagation in sPMCAb with respect to incubation time to disease, attack rate, and clinical signs (Table 2). These results indicate that in vitro propagation of a strain using partially deglycosylated PrPSc did not lead to an irreversible change of strain-specific features.
DISCUSSION

The current studies introduced a new PMCAb format referred to as dgPMCAb that utilizes partially deglycosylated substrate. Remarkably, relatively modest changes in PrP\textsuperscript{C} glycosylation status were found to be sufficient for controlling the selectivity of amplification of two transmissible PrP states formed within the same host. Although PMCAb selectively amplified PrP\textsuperscript{Sc}, dgPMCAb predominantly amplified atypical PrPres. Notably, in a previous study that employed PMCA, the stoichiometry of host PrP\textsuperscript{C} glycoforms was found to control the efficiency of priion amplification in a species-specific manner, where diglycosylated PrP\textsuperscript{C} was found to be required to propagate hamster strains (29). Considering that the ratio of PrP\textsuperscript{C} glycoforms varies in different brain regions and cell types (30–32), one can propose that different brain regions/cell types favor either PrP\textsuperscript{Sc} or atypical PrPres depending on the amount of un-, mono-, and diglycosylated PrP\textsuperscript{C} that is expressed in a particular region. This hypothesis remains to be tested in future studies.

Synthetic strains S05 and LOTSS generated in hamsters as a result of inoculation of recombinant PrP fibrils were shown to be a mixture of self-propagating states. Serial passage of both strains revealed a common evolutionary trend, which was characterized by elimination of atypical PrPres, emergence of PrP\textsuperscript{Sc}, and transformation of the neurological profile and PrP\textsuperscript{Sc} deposition pattern (27, 28). Notably, three serial passages were necessary for generating clinical transmissible spongiform encephalopathy disease in hamsters upon inoculation of amyloid recombinant PrP fibrils (27). In fact, despite deposition of large amounts of PrP\textsuperscript{Sc}, no clinical signs were observed in animals of LOTSS second passage. Remarkably, the evolutionary trend observed during three serial passages in animals was reproduced in sPMCAb reactions. Indeed, when brain material from LOTSS first passage was subjected to serial PMCAb prior to inoculation, clinical disease with the LOTSS-specific disease phenotype was observed at the second passage. These results illustrate that serial replication in PMCAb can accelerate the natural course of synthetic strain evolution.

Several atypical PrPres forms similar to those associated with the synthetic strains LOTSS and S05 were previously described in natural transmissible spongiform encephalopathy diseases including atypical bovine spongiform encephalopathy, scrapie, and Creutzfeldt-Jakob disease (33–35). The precise relationship between atypical PrPres and PrP\textsuperscript{Sc} remains to be elucidated. However, our work suggested that atypical PrPres, although structurally different from PrP\textsuperscript{Sc}, can give rise to PrP\textsuperscript{Sc} in seed events that appear to be stochastic (28). The finding that dgPMCAb-derived atypical PrPres contained small amounts of PrP\textsuperscript{Sc} suggests that PrP\textsuperscript{Sc} is amplified in dgPMCAb at low levels. This result is also consistent with the hypothesis that atypical PrPres can give rise to PrP\textsuperscript{Sc} in sPMCAb.

The utility of dgPMCA for studying natural atypical prion diseases remains to be demonstrated. If brain or tissue materials display atypical patterns of PK-resistant products, coupling of PMCAb with dgPMCAb might offer an in vitro approach for selective amplification of different self-propagating states present in a mixture. Furthermore, regardless of what is the origin of LMW products, dgPMCAb might be useful for strain typing. dgPMCA was able to detect conformational differences between those strains (for instance, between SSLOW and 263K) that give very similar if not identical PK resistance profiles in PMCAb.

Replication of 263K and HY in serial dgPMCAb dramatically transformed their PK resistance profile. In addition to PrP27–30, LMW PK-resistant products were always present in dgPMCAb-derived material. A similar transformation in the PK resistance profile was also found for synthetic strain SSLOW, which lacked atypical PrPres. Although the gel mobility of LMW products and atypical PrPres appeared to be alike, our results demonstrated that LMW bands were unrelated to atypical PrPres, but instead were generated from PrP\textsuperscript{Sc}.

Appearance of LMW products suggests that internal sites became more accessible to PK in dgPMCAb-derived PrP\textsuperscript{Sc} than in brain-derived PrP\textsuperscript{Sc}. An increase in accessibility could be due to reduced spatial interference between carbohydrate moieties
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...and PK in dgPMCAb-derived PrPSc and/or due to some structural changes in PrPSc as a result of its adaptation to partially deglycosylated PrPc. Although it is possible that limited glycosylation contributed in part to an increase in accessibility of the internal sites, nevertheless, no LMW products were reported in previous studies on PrPSc produced from unglycosylated substrate such as recombinant PrP or fully deglycosylated PrPc (36–38). Considering these data, it is likely that PrPSc replication in dgPMCAb involves an adaptation. Indeed, the gradual shift in mobility of SSLOW LMW products during dgPMCAb supports the adaptation hypothesis. It is noteworthy that previous studies using mass spectrometry identified several internal PK cleavage sites including residues 139, 142, and 154 in 263K and Drowsy brain-derived PrPSc upon treatment with high concentrations of PK and/or exposure to partially denaturing conditions (39, 40). Adaptation of PrPSc to dgPMCAb might involve changes in its quaternary structure leading to an increase in accessibility of internal sites. Nevertheless, the transformation in the PK resistance profile was fully reversible. When dgPMCAb-derived material was used to seed PMCAb, LMW products disappeared. Furthermore, as judged from animal bioassay, passage of 263K brain-derived materials through dgPMCAb did not change strain phenotype (Fig. 9 and Table 2). This result was consistent with the previous work by Piro et al. (37), in which prion strains amplified in PMCA with a fully deglycosylated PrPc substrate were shown to maintain their strain-specific properties.

With the finding of a reversible change in a strain-specific biochemical phenotype, it is interesting to consider phenotypic variations of a single genotype across a range of environments, known in population genetics as a norm of reaction. The concepts of norm of reaction and phenotypic plasticity were introduced in ecology and population genetics to describe variation in phenotype and the ability of an organism to change its phenotype, respectively, in response to changes in the environment. For instance, plants can acquire multiple morphologically distinct phenotypes within a single genotype to fit into a diverse range of environments. It is noteworthy that phenotypic plasticity is not attributed to mutations but to an intrinsic norm of reaction.

We believe that concepts analogous to phenotypic plasticity and norm of reaction could be applied to describe the behavior of prion strains. In the experiments on the replication of 263K, HY, or SSLOW in dgPMCAb, we observed structural plasticity as reflected by the ability of the strains to change their PK resistance profile in response to different degrees of substrate glycosylation, while retaining molecular features responsible for the strain-specific phenotype. The concepts of phenotypic plasticity and norm of reaction can also be useful for explaining the reversible changes in strain characteristics upon altering the replication environment, for instance, when brain-derived prions are replicated in PMCA and then transferred back to animals (18, 19, 23) or replicated in cultured cells and then back into animals (2, 5).

It has been proposed that each prion strain consists of a mixture of quasispecies or substrains (4). Quasispecies populations are dynamic; their composition changes in response to changes in the replication environment (2). It is not yet clear what molecular features are responsible for differences between quasispecies within each particular strain and whether these differences are entirely due to variation in PrPSc structure or due to the involvement of different cofactors (41–43). Recent work suggests that cellular lipids might be important for defining PrPSc strain-specific features (41). If cofactor environment is indeed important for defining strain-specific features, a strain shift might not have been predicted to occur during passage through dgPMCAb because the modification does not involve changes in cofactor environment. Studies that employed deep UV Raman spectroscopy revealed that highly uniform crystalline-like cross-β-structures can adopt multiple alternative fibril morphologies producing heterogeneous PrP fibril populations while preserving uniform cross-β-structures (44, 45). This result illustrates that structurally uniformity is possible despite highly heterogeneous morphological appearance.

The current work raised a number of questions about the need for a distinction between actual strain mutation and strain phenotypic or conformational plasticity. Should we attribute vast phenotypic variations, for example those observed in cell lines (2, 5), to actual strain mutations or to phenotypic plasticity due to replication in altered cellular environments? Is the term mutation applicable when someone deals with reversible or interconvertible changes between quasispecies or substrains? Considering that prion strain mutation has been employed for describing heritable change in disease phenotypes, should we rely on the same terminology for describing changes in strain characteristics that are expressed only within the specific environment of cultured cells or PMCA but reverse back to original strain characteristics when replicated in animals? What criteria should we use to define prion strain mutation? Future studies should address these questions.

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