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TECHNICAL REPORT

Role of prion protein glycosylation in replication of human prions by protein misfolding cyclic amplification

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Abstract

Prion diseases are transmissible neurological disorders associated with the presence of abnormal, disease-related prion protein (PrP^D). The detection of PrP^D in the brain is the only definitive diagnostic evidence of prion disease and its identification in body fluids and peripheral tissues are valuable for pre-mortem diagnosis. Protein misfolding cyclic amplification (PMCA) is a technique able to detect minute amount of PrP^D and is based on the conversion of normal or cellular PrP (PrP^C) to newly formed PrP^D, sustained by a self-templating mechanism. Several animal prions have been efficiently amplified by PMCA, but limited results have been obtained with human prions with the exception of variant-Creutzfeldt–Jakob-disease (vCJD). Since the total or partial absence of glycans on PrP^C has been shown to affect PMCA efficiency in animal prion studies, we attempted to enhance the amplification of four major sporadic-CJD (sCJD) subtypes (MM1, MM2, VV1, and VV2) and vCJD by single round PMCA using partially or totally unglycosylated PrP^C as substrates. The amplification efficiency of all tested sCJD subtypes underwent a strong increase, inversely correlated to the degree of PrP^C glycosylation and directly related to the matching of the PrP polymorphic 129 M/V genotype between seed and substrate. This effect was particularly significant in sCJDMM2 and sCJDVV2 allowing the detection of PK-resistant PrP^D ($resPrP^D$) in sCJDMM2 and sCJDVV2 brains at dilutions of 6×10^7 and 3×10^6. vCJD, at variance with the tested sCJD subtypes, showed the best amplification with partially deglycosylated PrP^C substrate reaching a $resPrP^D$ detectability at up to 3×10^{16} brain dilution. The differential effect of substrate PrP^C glycosylations suggests subtype-dependent PrP^C–PrP^D interactions, strongly affected by the PrP^C glycans. The enhanced PMCA prion amplification efficiency achieved with unglycosylated PrP^C substrates may allow for the developing of a sensitive, non-invasive, diagnostic test for the different CJD subtypes based on body fluids or easily-accessible-peripheral tissues.

Introduction

Human prion diseases are characterized by a variety of disease phenotype often exhibiting very different clinical presentations and disease durations [1–5]. Sporadic Creutzfeldt–Jakob disease (sCJD), which account for nearly 90% of all human prion diseases, displays at least five distinct subtypes associated with different disease phenotypes, and a number of mixed phenotypes [6], which may differ widely not only concerning clinical presentation but also disease duration. The early identification of these subtypes may become increasingly important since future treatments of human prion diseases are likely to be selective as for type and subtype of prion disease [7, 8].

Recently, great progress has been made on the diagnosis of prion disease by the testing of body fluids (blood and urine) or peripheral tissues (olfactory mucosa and skin) based on real time-quaking induced conversion (RT-QuIC) [9–11] and other methods that rely on advanced prion enrichment approaches [12–15]. However, none of these tests can identify type or subtype of sCJD on individual patients, which currently can be achieved only by brain tissue examination [4, 16].

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Protein misfolding cyclic amplification (PMCA), based on the in vitro conversion of normal or cellular PrP (PrP^C) to PrP^D, sustained by a self-templating mechanism [17] has shown an extraordinary competence to detect and faithfully replicate PrP^D from animal scrapie, capable of detecting a single molecule of PrP^D [18, 19]. However, amplification of PrP^D from human prion patients has been difficult [20–22]. Notable exceptions are represented by variant CJD (vCJD), a form of prion disease acquired from consumption of prion contaminated bovine meat [23] and, to a lesser extent, iatrogenic CJD, another type of acquired CJD related to medical or surgical interventions; in both these conditions PrP^D has been shown to amplify with high efficiency [22, 24, 25].

The achievement of high amplification efficiency by PMCA in body fluids or peripheral tissues from sCJD patients will likely allow for the accurate, non-invasive diagnosis of sCJD subtype early in the course, which will help plan the management and hopefully the future treatment of these patients.

Since several investigators reports have shown that glycan modification of mouse and hamster PrP^C, may affect PMCA efficiency [26–30], we tested substrates with glycan modified human PrP^C, as a preliminary attempt to improve the efficiency of PMCA of sCJD-PrP^D. For this purpose, we used a transgenic (Tg) mouse line expressing glycan-free human PrP^C-129M, generated in our lab, and Tg mouse lines expressing human PrP^C-129M and -129V, in conjunction with partial deglycosylation by PNGase F treatment in non-denaturing conditions. Our results showed a significant increase of PMCA amplification of sCJD and vCJD PrP^D strains when partially or totally deglycosylated human PrP^C as a substrate was used.

Materials and methods

Tissue samples

Frontal cortex from two cases each with sCJDMM1, sCJDMM2, sCJDVV1, and sCJDVV2 along with one case with vCJD were obtained from the National Prion Disease Pathology Surveillance Center (NPDPSC) of Case Western Reserve University, Cleveland, OH. Brain homogenates (BH), 10% w/v, were prepared on ice in conversion buffer (Dulbecco's phosphate-buffered saline 1×, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.05% digitonin, 1× protease inhibitor cocktail) with a cell disrupter Mini-beadbeater (BioSpec). Partially deglycosylated human PrP^C substrates were obtained by treating BH from Tg(HuPrPM) and Tg (HuPrPV) with 3200 U/ml of PNGase F in non-denaturing conditions for 1 h in a rotor at 37 °C. PNGase was not inactivated or removed from the substrates, as these treatments strongly affect the substrate efficiency in PMCA.

Animals

Three transgenic (Tg) mouse lines expressing human prion protein (PrP) were used. Tg(HuPrPM) and Tg(HuPrPV) expressed normal human PrP (PrP^C). TgNN6h line, generated in the Kong laboratory, expressed glycan-free human PrP-129M (HuPrPMGlyKO) at 0.6× the normal level (measured against wild type FVB mice) [21]. TgNN6h was generated by replacing the two asparagine residues for (N)-linked glycosylation at residues 181 and 197 with glutamine.

Protein misfolding cyclic amplification (PMCA)

PMCA was performed as previously described with minor modifications [17]. The BH from Tg(HuPrPM), Tg (HuPrPM)-PNGase treated, Tg(HuPrPV), Tg(huPrPV)-PNGase treated and TgNN6h were mixed in a 9:1 ratio with BH from the different sporadic CJD (sCJD) or variant CJD (vCJD) in the presence of 0.05% digitonin detergent (Sigma-Aldrich). The obtained BH 10% was serially diluted 1:10 (10^{-2}–10^{-N}, N depending on the subtype) with the respective substrates. Unseeded substrates were also processed by PMCA as negative control (in quadruplicate for each of the N PMCA experiments performed for seeded substrates, as specified). N PMCA experiments were performed for each subtype-substrate group as specified, testing 2 cases for every subtypes, with the exception of vCJD and MM2 with 129V + PNGase substrate, where single cases were analyzed. A single round of PMCA was carried out in a programmable sonicator (Qsonica Q500; Qsonica LLC, Newtown, CT) for 96 cycles. Each cycle consisted of 30 s of sonication and 29.5 min of incubation at 37 °C. One Teflon bead of 2.38 mm (McMaster-Carr, Los Angeles, CA) was added to each tube. The sonicator was operated at an amplitude optimized for each substrate. When the partially deglycosylated PrP^C substrates were used, an amplitude of 24 was selected. For fully glycosylated and fully unglycosylated PrP^C substrates, amplitudes of 28 and 26 were used, respectively.

Western blot

Aliquots of serially diluted BH, before and after PMCA treatment, were treated for 1 h at 40 °C with proteinase K (PK) (Roche Diagnostics) at the concentration of 200 µg/ml. At the end of PK digestion sample buffer 2× (Laemmli sample buffer, BioRad) was added 1:2 and the samples boiled for 10 min. Denatured samples were mixed 1:5 with pre-chilled methanol, vortexed and incubated at −20 °C for at least 2 h before centrifugation at 16,000 × g for 30 min at 4 °C. The pellets were resuspended in sample buffer and boiled for 10 min before loading. Protein samples were separated with Tris–glycine SDS-PAGE in 15% Criterion Tris–HCl polyacrylamide precast gels (Bio-Rad

Laboratories, Hercules, CA, USA) and transferred to Immobilon-P PVDF transfer membrane (EMD-Millipore, Billerica, MA, USA) for 2 h at 60 V, blocked with 5% nonfat dry milk in 0.1% Tween, 20 mM Tris-buffered saline, pH 7.5, and probed with the 3F4 antibody to PrP. The immunoreactivity was visualized by enhanced chemiluminescence (Pierce ECL 2, Fisher Scientific, Hampton, NH, USA) on Kodak BioMax Light films (Eastman Kodak Co., Rochester, NY, USA).

Maximum amplification of PK-resistant disease-associated PrP ($resPrP^D$) detected after PMCA treatment was determined by limit dilution and expressed as the highest dilution of the seed allowing detection of $resPrP^D$ (detection limit). Amplification efficiency was expressed as the ratio of $resPrP^D$ concentrations, quantified by densitometry, detected in PMCA-treated vs PMCA-untreated samples, multiplied by the increase of brain dilution detectability [($resPrP^D$ PMCA+/$resPrP^D$ PMCA−) × limit dilution increase]. Densitometry was performed on the scanned WBs images by UN-SCAN-IT software (Silk Scientific).

PrP^D quantification

Concentration of vCJD-$resPrP^D$ was obtained comparing, by western blotting, brain homogenate aliquots with serially diluted recombinant PrP (recPrP) 23-231. Densitometry was performed on the scanned WBs images by UN-SCAN-IT software and $resPrP^D$ concentration calculated by extrapolation of the recPrP calibration curve.

Statistical analysis

Statistical analyses were performed with analysis of variance (one-way ANOVA) of amplifications obtained with the different substrates for either each or all CJD subtypes, as specified, followed by Tukey's multiple comparison test. For the comparison of subtype-specific PMCA efficiency, one-way ANOVA was also calculated on the groups formed by the amplifications, with the same substrate, of the different subtypes, followed by Tukey's test.

Results

Quantification of PrP^C in the different transgenic mice lines

Analysis of brain PrP^C concentration in the three Tg mice lines showed a virtual equivalence between Tg(HuPrPV) and TgNN6h (with glycan-free human PrP-129M), 1.1× and 1× respectively, and a 4.1× higher PrP^C concentration in Tg (HuPrPM) (Supplementary Fig. 1).

PMCA with fully glycosylated human PrP^C substrates

Treatment by PMCA (single round, as in the entire study) of PrP^D species associated with different subtypes of sCJD showed great variability in their propensity to amplify, depending on sCJD subtype and 129M/V polymorphism of the PrP^C substrate (Figs. 1a and 2; Tables 1 and 2). Using the PrP^C-129M substrate, PK-resistant PrP^D ($resPrP^D$) associated with sCJDMM1 and sCJDVV2 was amplified 3.19- and 9.64-fold, respectively, while the levels of sCJDVV1 and sCJDMM2 $resPrP^D$ exhibited a paradoxical decrease, up to 3-fold for sCJDMM2 (Figs. 1a and 2; Tables 1 and 2). With the PrP^C-129V substrate, $resPrP^D$ decreased by almost 4 times for sCJDMM2, remained virtually unchanged for sCJDMM1 and sCJDVV1, and increased 40-fold for sCJDVV2. This last amplification allowed the detectability of sCJDVV2 at up to 2.62×10^3 brain dilution, the highest amplification of all sCJD subtypes using fully glycosylated PrP^C as substrate (Figs. 1a and 2; Tables 1 and 2). Much higher amplification was achieved, using the PrP^C-129M substrate, for vCJD. In this case, $resPrP^D$ was detectable at up to 5×10^8 dilution, an amplification of 3.59×10^5-fold (Figs. 1b and 2; Tables 1 and 2).

PMCA with partially deglycosylated PrP^C substrate

The use of partially deglycosylated PrP^C substrates, obtained by PNGase F digestion (Supplementary Fig. 2), resulted in a significant increase of $resPrP^D$ PMCA amplification of all CJD seeds tested with the PrP-129 homologous substrate. This effect was particularly evident for sCJDMM2 and sCJDVV2 where, using the respective seed-substrate pairing, $resPrP^D$ was detectable at up to 10^6 brain dilution, resulting in a net amplification of 2.22×10^3 and 54.63-fold respectively (Figs. 1 and 2; Tables 1 and 2). In vCJD $resPrP^D$ amplification were unexpectedly high, reaching the detectability at 3.37×10^{16} brain dilution with a net amplification of approximately 10 billion-fold over the amplification with the normally glycosylated PrP^C-129M substrate (Figs. 1b and 2; Tables 1 and 2). Quantification of $resPrP^D$ in vCJD seed showed a concentration of 24.9 µg $resPrP^D$/mg brain. Estimating a weight of around 4.1×10^{-20} g for a single vCJD-$resPrP^D$ molecule (average of the 3 glycoforms), 6.1 $resPrP^D$ molecules is expected to be present in 100 µl (the volume used in PMCA analysis) of 10^{16}-fold diluted brain (Supplementary Fig. 4).

PMCA with unglycosylated PrP^C substrate

When PMCA was performed with the totally unglycosylated PrP^C-129M substrate (MGlyKO) from the TgNN6h mice, $resPrP^D$ amplification was further increased for both

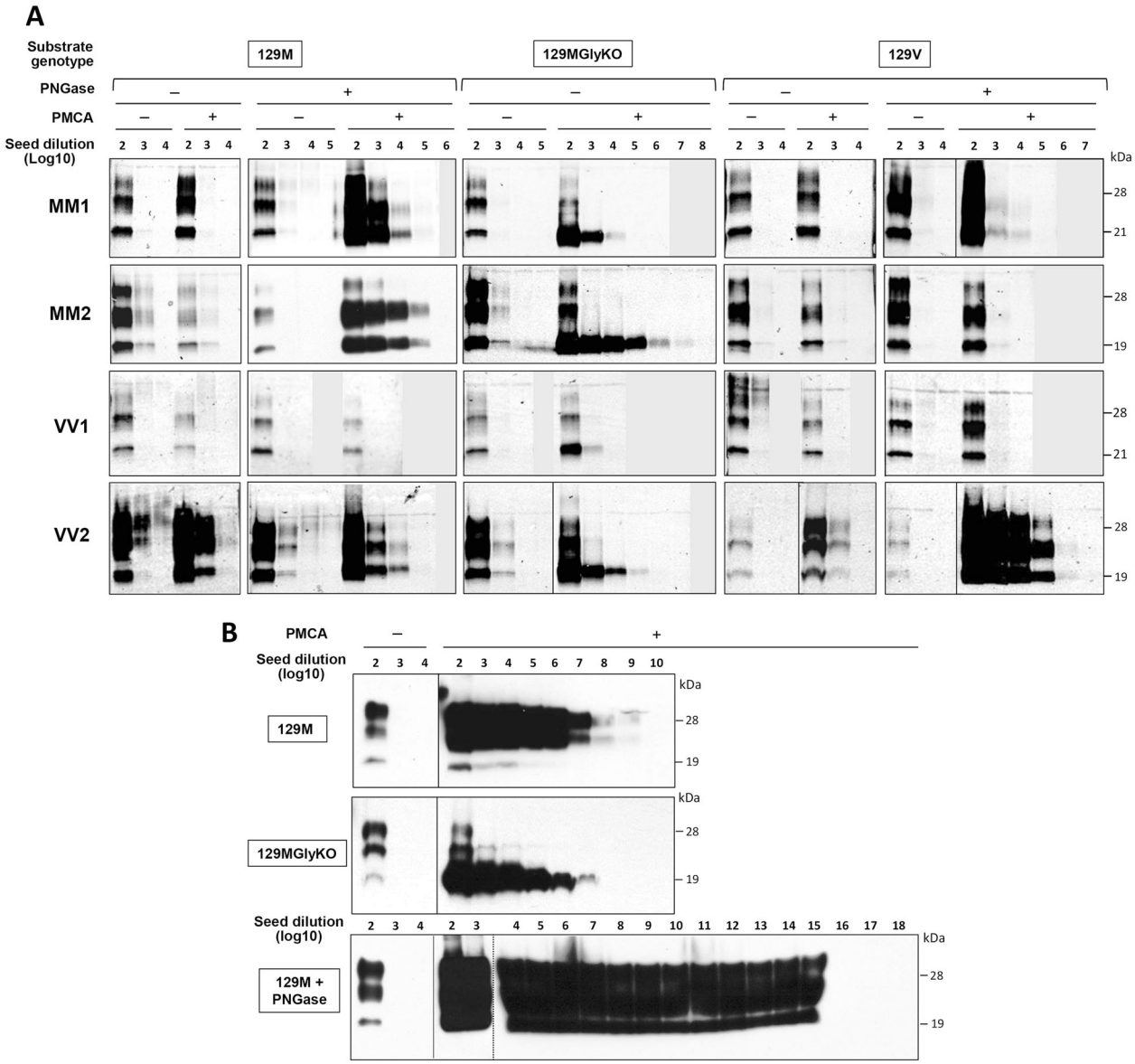

Fig. 1 Amplification of resPrP^{D} from different sCJD subtypes and vCJD by PMCA with five distinct substrates. **a** Immunoblots of PK-resistant disease-related prion protein (resPrP^{D}) from the indicated sCJD subtypes brain homogenates (BH) that, used as seed, were submitted to limit dilution of carrier brain homogenates (seed dilution, expressed as log10, 2–8) and were analyzed before and after PMCA treatment. PMCA was performed with five substrates: fully glycosylated PrP, 129M and 129V, (PNGase −); partially deglycosylated PrP, 129M and 129V (PNGase +); totally unglycosylated PrP (129MGlyKO). The concentration variability of PrP^{D} in some PMCA-untreated samples is due to different preparations and/or different cases. Every panel shows samples run in a single gel; black lines refer to panel cropped to size. **b** vCJD; procedure as in (**a**) but with the three indicated substrates and log10, 2–18. Lanes separated by the dotted line in the middle panel were run in two separate gels due to high number of samples

sCJDMM1 and sCJDMM2 subtypes. The enhancement was marginal for sCJDMM1 (1.09-fold) but significant for sCJDMM2 (15.54-fold), achieving resPrP^{D} detectability at up to 5.5×10^{7} brain dilutions (Figs. 1a and 2; Tables 1 and 2).

The lack of glycan-free human PrP^{C}-129V substrate prevented examination of its effect on resPrP^{D} amplification for sCJDVV1 and sCJDVV2. However, a distinct increase of amplification efficiency was also observed with the glycan-free PrP^{C}-129M (TgNN6h) when compared with the partially deglycosylated PrP^{C}-129M substrate. For sCJDVV1 the increment was approximately 3.4-fold, but it is still only about a half of the amplification obtained with partially deglycosylated PrP^{C}-129V (Fig. 2 and Table 1). Nonetheless, the detection limit was paradoxically higher than that with partially deglycosylated 129V (Fig. 1 and Table 2). sCJDVV2 also showed an amplification increase of 2.28-fold, but it is still 24 times lower than that obtained with partially deglycosylated PrP^{C}-129V substrate. (Figs. 1a

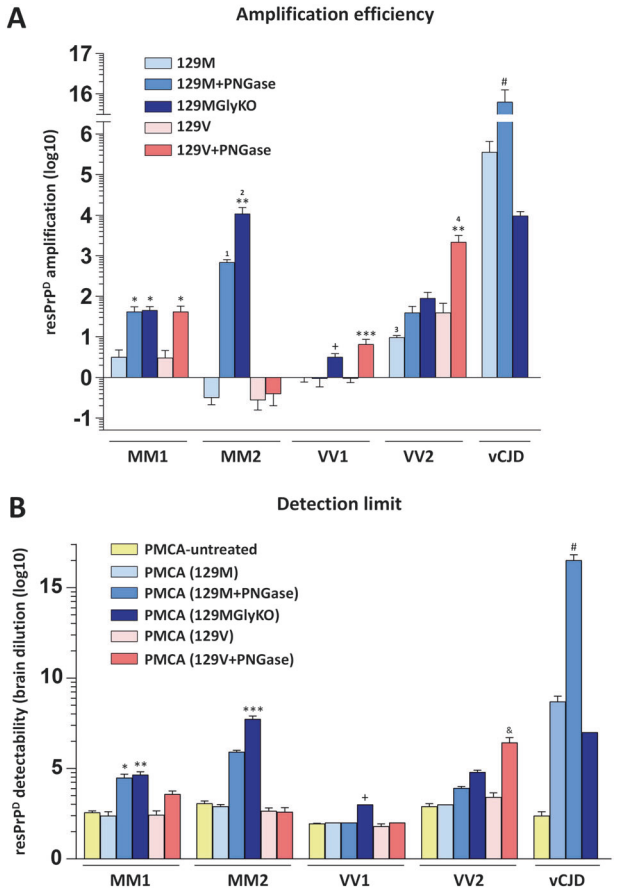

Fig. 2 Bar graph of amplification efficiency and limit detectability of resPrP^D from four sCJD subtypes and vCJD by PMCA using distinct substrates. The five substrates were: (1) fully glycosylated PrP denoted 129M and 129V; (2) partially deglycosylated PrP, 129M and 129V denoted 129M + PNGase and 129V + PNGase; (3) completely unglycosylated PrP denoted 129MGlyKO. Only 129M, 129M + PNGase, and 129MGlyKO were used for vCJD. Values are expressed as mean ± SEM. At least a PMCA duplicate on 2 cases was performed in all sCJD subtypes, while a single case was tested in vCJD and MM2 with 129V + PNGase substrate. The number of PMCA experiments (4–8) performed for each group is specified in Tables 1 and 2. **a** Amplification efficiency. Statistical significance: $*p < 0.0001$ vs PMCA-untreated (value 0) and $p < 0.02$ vs 129M and 129V; $**p < 0.0001$ vs PMCA-untreated and every substrate; $***p < 0.001$ vs PMCA-untreated and every substrate; $^{+}p < 0.05$ vs PMCA-untreated; $^{\#}p < 0.0001$ vs PMCA-untreated and every substrate of all tested CJD subtype; ^{1}129M + PNGase of MM2 is statistically different from that of MM1, MM2, and VV1, $p < 0.0001$; ^{2}129MGlyKO of MM2 is statistically different from that of MM1, VV2, and VV1, $p < 0.002$; ^{3}129M of VV2 is statistically different from that of MM1, $p < 0.005$ and from that of MM2 and VV1, $p < 0.002$; ^{4}129V + PNGase of VV2 is statistically different from that of MM1 and MM2, $p < 0.02$, and VV1, $p < 0.005$. **b** Detection limit. Statistical significance: $*p < 0.02$ vs PMCA-untreated; $**p < 0.02$ vs PMCA-untreated and every substrate, with the exception of 129M + PNGase; $***p < 0.001$ vs PMCA-untreated and every substrate; $^{+}p < 0.0001$ vs PMCA-untreated and every substrate; $^{\&}p < 0.01$ vs PMCA-untreated and every substrate; $^{\#}p < 0.0001$ vs PMCA-untreated and every substrate of all tested CJD subtype. For description, see "Results"

Table 1 Amplification efficiency of PK-resistant disease-related PrP (resPrP^D) from sCJD subtypes and vCJD using PrP glycosylation-variant PrP substrates

Seed	Substrate							
	129M (a)	129M + PNGase (b)	Incr. (b)/(a)	129MGlyKO (c)	Incr. (c/a)	129V (d)	129V + PNGase (e)	Incr. (e)/(d)
sCJDMM1	3.19 ± 1.52 (6)	41.73 ± 13.39 (7)*	13.08	45.48 ± 9.76 (5)*	14.26	3.05 ± 1.57 (5)	41.64 ± 15.59 (6)*	13.65
sCJDMM2	0.31 ± 0.10 (5)	687.10 ± 110.70 (6)[a]	2.22×10^3	$1.07 \times 10^4 \pm 4.62 \times 10^3$ (4)**,[b]	3.45×10^4	0.27 ± 0.12 (5)	0.39 ± 0.19 (3)	1.44
sCJDVV1	0.99 ± 0.22 (5)	0.93 ± 0.35 (4)	0.94	3.17 ± 0.67 (5)$^{+}$	3.20	0.94 ± 0.19 (5)	6.57 ± 2.09 (7)***	6.99
sCJDVV2	9.64 ± 1.07 (4)[c]	39.24 ± 17.50 (6)	4.07	89.29 ± 34.88 (8)	9.27	39.72 ± 27.23 (5)	$2.17 \times 10^3 \pm 960.8$ (4)**,[d]	54.63
vCJD	$3.59 \times 10^5 \pm 2.95 \times 10^5$ (2)	$6.22 \times 10^{15} \pm 6.22 \times 10^{15}$ (3)$^{\#}$	2.6×10^{10}	$9.64 \times 10^3 \pm 2.53 \times 10^3$ (2)	0.03	NA	NA	NA

Amplification efficiency was determined with the formula [(resPrP^D PMCA+/resPrP^D PMCA−) × limit dilution increase]. Mean ± *SEM* (*N*: number of experiments). Incr.: increase of resPrP^D amplification efficiency when amplifications, obtained with normal (a and d) and abnormal (b, c, and e) glycosylated PrP substrates, were compared. Statistical significance: $*p < 0.0001$ vs PMCA-untreated (value 0) and $p < 0.02$ vs 129M and 129V; $**p < 0.0001$ vs PMCA-untreated and every substrate; $***p < 0.001$ vs PMCA-untreated and every substrate; $^{+}p < 0.05$ vs PMCA-untreated; $^{\#}p < 0.0001$ vs PMCA-untreated and every substrate of all tested CJD subtype; [a]129M + PNGase of MM2 is statistically different from that of MM1, MM2, and VV1, $p < 0.0001$; [b]129MGlyKO of MM2 is statistically different from that of MM1, VV2, and VV1, $p < 0.002$; [c]129M of VV2 is statistically different from that of MM1, $p < 0.005$ and from that of MM2 and VV1, $p < 0.002$; [d]129V + PNGase of VV2 is statistically different from that of MM1 and MM2, $p < 0.02$, and VV1, $p < 0.005$

Table 2 Detection limit of PK-resistant disease-related prion protein ($resPrP^D$) from sCJD subtypes and vCJD with or without PMCA treatment using glycosylation-variant PrP substrates

Seed	PMCA-untreated	PMCA treated (different substrates)				
		129M	129M + PNGase	129MGlyKO	129V	129V + PNGase
sCJDMM1	$3.76 \times 10^2 \pm 7.91 \times 10$ (29)	$2.5 \times 10^2 \pm 1.5 \times 10^2$ (6)	$3.06 \times 10^4 \pm 1.8 \times 10^4$ (7)*	$4.6 \times 10^4 \pm 2.2 \times 10^4$ (5)**	$2.8 \times 10^2 \pm 1.8 \times 10^2$ (5)	$3.85 \times 10^3 \pm 8.86 \times 10^3$ (6)
sCJDMM2	$1.2 \times 10^3 \pm 4.27 \times 10^2$ (23)	$8.2 \times 10^2 \pm 1.8 \times 10^2$ (5)	$8.5 \times 10^5 \pm 1.5 \times 10^5$ (6)	$5.5 \times 10^7 \pm 2.6 \times 10^7$ (4)***	$4.6 \times 10^2 \pm 2.21 \times 10^2$ (5)	$4 \times 10^2 \pm 3 \times 10^2$ (3)
sCJDVV1	$8.96 \times 10 \pm 5.75$ (26)	$1 \times 10^2 \pm 0$ (5)	$1 \times 10^2 \pm 0$ (4)	$1 \times 10^3 \pm 0$ (5)$^+$	$6.4 \times 10 \pm 2.21 \times 10$ (5)	$1 \times 10^2 \pm 0$ (7)
sCJDVV2	$7.97 \times 10^2 \pm 3.64 \times 10^2$ (27)	$1 \times 10^3 \pm 0$ (4)	$8.5 \times 10^3 \pm 1.5 \times 10^3$ (6)	$6.51 \times 10^4 \pm 1.7 \times 10^4$ (8)	$2.62 \times 10^3 \pm 1.85 \times 10^3$ (5)	$2.78 \times 10^6 \pm 2.42 \times 10^6$ (4)$^{\&}$
vCJD	$2.5 \times 10^2 \pm 1.5 \times 10^2$ (7)	$5.05 \times 10^8 \pm 4.95 \times 10^8$ (2)	$3.37 \times 10^{16} \pm 3.32 \times 10^{16}$ (3)$^{\#}$	$1 \times 10^7 \pm 0$ (2)	NA	NA

Highest dilution of brain homogenate allowing $resPrP^D$ detection. Values: mean brain dilutions ± *SEM* (*N*: number of experiments). Statistical significance: *$p < 0.02$ vs PMCA-untreated; **$p < 0.02$ vs PMCA-untreated and every substrate, with the exception of 129M + PNGase; ***$p < 0.001$ vs PMCA-untreated and every substrate; $^+p < 0.0001$ vs PMCA-untreated and every substrate; $^{\&}p < 0.01$ vs PMCA-untreated and every substrate; $^{\#}p < 0.0001$ vs PMCA-untreated and every substrate of all tested CJD subtype

and 2; Tables 1 and 2). Unexpectedly, vCJD showed a major reduction of amplification vs that obtained with both partially glycosylated PrP^C (6×10^{11}-fold) and totally glycosylated PrP^C (37-fold) (Figs. 1b and 2; Tables 1 and 2).

No spontaneous resPrP has been detected, after PMCA, in any unseeded-substrate (Supplementary Fig. 3).

Discussion

Our findings show that the extent of glycosylation of the PrP^C, used as a substrate in PMCA, strongly impacts the amplification of $resPrP^D$ from all sCJD subtypes tested and vCJD. Furthermore, diminished or total lack of PrP^C glycosylation appears to strengthen the requirement for genotype matching at residue 129 between seed and substrate. For sCJD, we observed that the amplification inversely correlated to the degree of PrP^C glycosylation, reaching the highest levels with glycan-free PrP^C-129M in sCJDMM1 and sCJDMM2, and with partially deglycosylated PrP^C-129V (the only glycan variant available with 129V genotype) for sCJDVV1 and sCJDVV2 subtypes.

At variance with this pattern, vCJD $resPrP^D$ amplification, which is already very high with the fully glycosylated PrP^C substrate, further increased by 10 logs with partially deglycosylated PrP^C but decreased, when using the glycan-free PrP^C substrate, to a level even lower than that achieved with the normally glycosylated substrate.

The observed effect of glycan-free PrP^C-129M is probably underestimated in our study as the PrP^C level in this Tg mouse line (TgNN6h) is only 25% of that in the Tg line expressing fully glycosylated PrP^C-129M, Tg(HuPrPM). A possible direct effect of the PrP mutation of this Tg line (TgNN6h) on the PMCA efficiency cannot also be ruled out.

The variability of $resPrP^D$ concentration observed in some samples (different cases and/or different tissue sampling) from the same subtype rendered more difficult the analysis of statistically differences using the limit detection method. However, the use of the densitometry-based method (amplification efficiency), independent from the different PrP^D starting concentrations, showed significant subtype-specific PMCA properties. In addition to the above discussed peculiar PMCA characteristics of vCJD, our findings include the highest amplification efficiency of MM2 with totally unglycosylated 129M vs all tested subtypes and partially deglycosylated 129M vs sCJD subtypes. Furthermore, VV2 showed the best amplification efficiency of all sCJD subtypes with fully glycosylated 129M and with the 129V substrates.

Previous studies have shown that the presence or absence of glycans in mouse or hamster PrP^C may positively or negatively affect the transmission [31, 32] and amplification by PMCA of different prion strains [26–30, 33]. Those

observations suggest an important strain-specific role of host PrP^C glycans in PrP^D replication and infectivity. Similarly, preliminary data on sCJD bioassays in mice expressing glycan-free human PrP-129M (TgNN6h) have shown prominent reduction of incubation time associated with increased severity of PrP^D-related histopathology after inoculation with sCJDMM2 but not of sCJDMM1 (Cracco et al., 90th annual meeting of American Association of Neuropathologists, 2014, and Kong et al., unpublished data).

The mechanism underpinning the observed effect of PrP^C glycosylation in PMCA is not clear. It may lie in the stabilizing effect of glycans on the PrP^C structure, making it more resistant to misfolding; or in the effect of the glycans steric hindrance on the strain-specific PrP^C–PrP^D interaction, consequently facilitating or hindering the specific conversion. The markedly different effect observed in sCJD and vCJD would favor the second hypothesis, also considering the typically distinctive glycosylation ratios of these two forms of CJD-PrP^D. Previous studies on scrapie strains, described an important role of sialylated N-linked glycans on PrP^C–PrP^D conversion, with significant increase of the PrP^D replication rate by PMCA after substrate desialylation [34, 35]. The authors attributed this effect to constraints generated by electrostatic repulsion between terminal sialic residues on N-linked glycans. If and how the results obtained in our study with deglycosylated substrates are related to this effect remains to be determined.

The greatest increases in $resPrP^D$ amplification that we obtained for sCJDMM2 and sCJDVV2 in a single round of PMCA, using partially or fully-deglycosylated PrP^C substrates, reached levels comparable to that obtained for vCJD amplified with a normal PrP^C substrate (Table 2). Therefore, these PMCA conditions may allow for the detection and electrophoretic profile identification of $resPrP^D$ in body fluids or easily accessible peripheral tissues from patients with sCJDMM2 and possibly sCJDVV2, especially if using the eventual Tg mice expressing glycan-free PrP^C-129V and after performing PrP^D enrichment procedures of the body fluids [36]. Moreover, knowing the PrP 129 genotype of the patient, the specific different detection limit might be used to distinguish patients with sCJDMM2 or -VV2 from those with -MM1 and -VV1. Since the amplification here reported was obtained in a single round of PMCA (96 cycles), the use of serial rounds of PMCA that greatly increase the amplification efficiency [18], might allow the direct detection of $resPrP^D$ of sCJDMM1 and -VV1 as well. These two subtypes, together with sCJDMM2 and sCJDVV2, account for nearly 90% of all cases of sCJD [37].

The vCJD $resPrP^D$ detection in brain diluted 3.4×10^{16}-fold after a single round of PMCA with partially deglycosylated PrP^C, is far higher than that previously reported of around 10^7 and 10^{11}-fold obtained with classical or modified PMCA conditions, respectively [24, 25, 38]. Consequently, it is reasonable to speculate that the use of PMCA with partially deglycosylated PrP^C would afford the detection of $resPrP^D$ in vCJD urine and blood, which have estimated $resPrP^D$ concentrations equivalent to vCJD brain diluted 10^{12}–10^{13} and 10^9-fold, respectively [24, 38], in a single round of PMCA rather than the 3–4 rounds needed in the original method described by Soto and colleagues [24, 38].

Against this backdrop, PMCA with glycan variant PrP^C substrates might provide the platform, when combined with PrP codon 129 genotyping, for a diagnostic test suitable to identify subtypes of CJD in the living patient.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The human samples were provided by NPDPSC. All procedures were performed under protocols approved by the Institutional Review Board of University Hospitals of Cleveland and as per regulations of the Declaration of Helsinki. All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under protocols #2016-0051 approved by the Institutional Animal Care and Use Committee of the Case Western Reserve University.

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