**In Vitro** Amplification of Misfolded Prion Protein Using Lysate of Cultured Cells

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**Abstract**

Protein misfolding cyclic amplification (PMCA) recapitulates the prion protein (PrP) conversion process under cell-free conditions. PMCA was initially established with brain material and then with further simplified constituents such as partially purified and recombinant PrP. However, availability of brain material from some species or brain material from animals with certain mutations or polymorphisms within the PrP gene is often limited. Moreover, preparation of native PrP from mammalian cells and tissues, as well as recombinant PrP from bacterial cells, involves time-consuming purification steps. To establish a convenient and versatile PMCA procedure unrestricted to the availability of substrate sources, we attempted to conduct PMCA with the lysate of cells that express cellular PrP (PrPC). PrPSc was efficiently amplified with lysate of rabbit kidney epithelial Rk13 cells stably transfected with the mouse or Syrian hamster PrP gene. Furthermore, PMCA was also successful with lysate of other established cell lines of neuronal or non-neuronal origins. Together with the data showing that the abundance of PrPSc in cell lysate was a critical factor to drive efficient PrPSc amplification, our results demonstrate that cell lysate in which PrPSc is present abundantly serves as an excellent substrate source for PMCA.

**Introduction**

Conformational conversion of the α-helix rich cellular prion protein (PrPC) to the β sheet rich scrapie prion protein (PrPSc) is the major biochemical event that characterizes prion diseases [1]. The protein-only hypothesis postulates that prion replication is the major biochemical event that characterizes prion diseases [1]. The protein-only hypothesis postulates that prion replication is the major biochemical event that characterizes prion diseases [1]. Protein misfolding cyclic amplification (PMCA) is an assay that mimics the PrPSc propagation process under cell-free conditions. This method amplifies misfolded PrP by converting PrPSc to PrPSc during incubation with periodic sonication [4]. PrPSc generated by PMCA is infectious in wild-type animals [5] and can be indefinitely propagated with preserved properties of the original PrPSc [5–7]. PMCA recapitulates the species barrier of prion transmission [8–11], prion strain interference [7], and de novo generation of prions [12,13]. Furthermore, PMCA is quite useful in studying the cofactors that influence PrP conversion [14–24], and in detecting PrPSc from biological samples of humans and animals [25–37]. PMCA has contributed to a number of important perspectives in prion biology, however, its conventional application to certain investigations still faces a few challenging problems. One of these problems is associated with the source of PMCA substrate. PMCA was originally designed to use brain homogenate derived from healthy animals that contains an excess amount of PrPSc, which a minute amount of prion-infected brain material, the source of PrPSc, was diluted [4]. This prototypic method has evolved to use the lipid raft fractions of the plasma membrane as the source for PrPSc [23,30,39] because PrP conversion occurs at the caveole-like membrane domains of neuronal cells [40–42]. Recently, PrPSc purified from brain tissue or cultured mammalian cells [19,43] and recombinant PrP expressed in bacterial cells [30,44,45] have replaced brain material for PMCA. Crude brain homogenate and the lipid raft fractions of the membrane provide a comprehensive set of components required for PMCA including a cofactor, while purified PrPSc or recombinant PrP offers defined minimal substrates. However, availability of brain material from certain species or transgenic animals carrying the PrP gene with certain mutations and polymorphisms is often limited. Alternatively, preparation of the substrates by expression/purification of native PrPSc from animal tissues and cell lines, as well as recombinant PrP from bacterial cells, requires additional, laborious steps. Thus, it is
necessary to establish a convenient alternative that overcomes aforementioned drawbacks of the current PMCA method.

In this study, we used cell lysate of cultured mammalian cell lines in PMCA reactions. Lysate of cultured cells has not been used as a substrate source for PMCA and it has been considered incapable of supporting PrPSc formation in PMCA unless complemented with brain homogenate that may include a cofactor for PrP conversion [6,46]. Based on our recent observation that PrPSc abundance is critical for robust PrPSc propagation in PMCA [21], we performed PMCA with PrP-expressing cell lysates in which the level of PrPSc was equivalent to wild type brain material. Here, we show that PMCA replication of mouse and hamster-adapted PrPSc using cell lines that express murine and hamster PrPSc, respectively.

Results

Estimation of the PrPSc level in cell lines

We established RK13 cells that express the full-length mouse and Syrian hamster PrP open reading frames, designated RK13MoPrP and RK13SHaPrP. We compared the level of PrPSc in RK13MoPrP to that of FVB/N wild type brain homogenate and several cell lines: N2a, SMB-PS, NIH 3T3, CRBL, and Hpl-3-4 cells (Fig. 1A). Western blot analysis indicated that RK13MoPrP cells expressed PrPSc at a higher level than other PrPSc-expressing cell lines. However, the abundance of PrPSc in RK13MoPrP cells was lower than wild type mouse brain, but similar to RK13MoPrP-gag cells in which the retroviral gag gene was coexpressed with mouse PrP. Among previously established cell lines, the level of PrPSc in N2a, SMB-PS, and CRBL was similar to each other, but greater compared to NIH 3T3. RK13, RK13 vector, and Hpl 3-4 lacked PrPSc expression. RK13SHaPrP cells expressed PrPSc at a lower level than wild type Syrian hamster brain.

Next, we investigated whether PrPSc expressed in our newly established RK13 cell line colocalized to lipid rafts in the plasma membrane as observed in nature. Immunofluorescence microscopy revealed that the location of PrPSc expression in RK13MoPrP cells was at the cell surface and colocalized with the glycosphingolipid GM1, a marker for lipid rafts (Fig. 1B). This suggests that PrPSc expressed in RK13MoPrP cells was processed normally and localized as usually found in nature.

PMCA propagation of PrPSc is supported using RK13MoPrP and RK13MoPrP-gag cell lysate

RK13MoPrP cell lysate was applied to PMCA for PrPSc amplification. Similar levels of PrPSc from RK13MoPrP cells and murine brain were used in PMCA (Fig. 1A). The PrPSc in RK13MoPrP cell lysate was converted into PrPSc when seeded with RML prions (Fig. 2A, top panel). This result was similar to PMCA using wild type brain homogenate (Fig. 2A, bottom panel). As expected, PrPSc of RML (Rocky Mountain Laboratory) prions was not propagated in the absence of PrPSc when lysate of uninfected RK13 cells was used in PMCA (Fig. 2A, second panel). Normal brain homogenate did not induce spontaneous PrPSc formation from RK13MoPrP cell lysate (Fig. 2A, third panel). In serial PMCA, RK13MoPrP cell lysate was able to support continuous propagation of PrPSc (Fig. 2B).

To investigate if PrPSc abundance in cell lysate influences PMCA conversion of PrPSc, we conducted PMCA with RK13MoPrP cell lysate diluted 1:10 in lysate of normal RK13 cells that do not express PrPSc. Consistent with our previous PMCA studies with brain material [21], the amount of PrPSc present in cell lysate dictated the level of PrPSc generation in PMCA. PrPSc amplification with diluted RK13MoPrP cell lysate was less efficient than amplification with undiluted substrate (Fig. 3). Spontaneous generation of PrPSc in PMCA was not observed when normal brain material was used as the seeding source (Fig. 3).

Because expression of the human immunodeficiency virus-1 (HIV-1) gag gene in cultured cells promotes formation of PrPSc in the cell culture models of prion disease [47], we assessed whether HIV-1 Gag influences in vitro amplification of PrPSc. To address this, we performed PMCA using RK13MoPrP-gag cell lysate as a substrate and PrPSc from RML prions as seeds. We failed to detect a significant difference in PrPSc conversion efficiency using PMCA between RK13MoPrP-gag and RK13MoPrP cell lysates (Fig. 4), suggesting that HIV-1 Gag does not affect PrPSc conversion.

Lysate of both neuronal and non-neuronal cell lines supports PMCA generation of PrPSc

To assess the ability of lysate derived from previously established cell lines to support PMCA, we prepared cell lysate in which PrPSc was concentrated to the same level as wild type mouse brain material (Fig. 1A). The cell lines from diverse origins were chosen: N2a neuroblastoma cells exhibit characteristics of neurons [48]; SMB-PS cells derived from scrapie-infected mouse brain but cured by in vitro treatment with pentosan sulfate are originated from the mesenchymal lineage [49]; CRBL cells derived from the cerebellum of p53 null mice show expression of both glial and neuronal markers [50]; and NIH 3T3 cells feature the characteristics of common fibroblasts [51]. All of the tested lysates supported PrPSc propagation in PMCA (Fig. 5), suggesting that PrPSc conversion is not exclusively dependent on the neuronal cells.

The efficiency of PMCA formation of PrPSc varies upon the cellular source of PrPSc

We compared the efficiency of PMCA generation of PrPSc using cell lysate from different sources. Based on the densitometric analysis of the Western blots, we plotted the normalized PrPSc levels of each pre- and post-PMCA sample. The PrPSc levels amplified with RK13MoPrP and N2a cell lysate were greater than those generated by wild type brain homogenate, but less than those generated by Tg(MoPrP)4112 [21] brain homogenate (Fig. 6A and B). The PrPSc levels amplified with CRBL and NIH3T3 cell lysate were almost identical with those generated by wild type brain homogenate (Fig. 6C). The SMB-PS cell lysate was not obviously better than wild type brain homogenate or cell lysate of other cell types (Fig. 6B). To further investigate the efficacy of RK13MoPrP and N2a cell lysate in generating PrPSc, the fold increase of newly synthesized PrPSc was plotted. The rates of PrPSc generation by RK13MoPrP and N2a cell lysate were intermediate between those obtained with Tg(MoPrP)4112 [21] brain homogenate (Fig. 6D). Similar analysis for cell lysate of other cell lines indicated that the rate of PrPSc formation resembled that of wild type brain homogenate (data not shown).

PMCA propagation of PrPSc is supported using lysate of RK13 cells that express Syrian hamster PrPSc

To address that cell lysate-based PMCA is not restricted to murine PrPSc and murine-adapted scrapie prions, we performed PMCA using Syrian hamster PrPSc and the hyper (HY) and drowsy (DY) strains of hamster-adapted transmissible mink encephalopathy (TME) prions [52]. PrPSc from either HY or DY strains was successfully amplified in PMCA using RK13SHaPrP cell lysate (Fig. 7A). The strain-specific migration of HY and DY PrPSc was similar between brain-derived and PMCA-generated material.
Fig. 7B). In particular, HY was more efficiently amplified than DY in cell lysate-based PMCA (Fig. 7B), which corresponds to the results of PMCA studies that used Syrian hamster brain homogenate and bioassays of HY and DY in Syrian hamsters [7].

**Discussion**

The current study demonstrates that cell lysate with concentrated PrP<sup>C</sup> allowed robust PMCA of PrP<sup>Sc</sup> from multiple strains and species. The ability of cell lysate to support PrP<sup>Sc</sup> formation in PMCA is comparable to that of wild type brain material. This result suggests that cell lysate can replace animal organ-derived material for in vitro PrP<sup>Sc</sup> amplification without compromising PrP conversion efficiency.

Previously, cultured cell lysate was shown to support extremely low levels of PrP<sup>Sc</sup> formation [46]. Moreover, PMCA with lysate of animal cells has been reported to require compensation with PrP<sup>Sc</sup>-containing brain material for adequate amplification [6], suggesting that additional factors derived from the brain material are required to properly amplify PrP<sup>Sc</sup> using cell lysate. In contrast to these results, our study demonstrated that cell lysate is sufficient to support PrP<sup>Sc</sup> amplification, although inclusion of the “auxiliary factors” in cell lysate remains to be elucidated. The discrepancy between previous and current results may be due to the abundance of PrPC in PMCA. As opposed to the previous studies using cell lysate with the endogenous level of PrP<sup>C</sup>, our study exploited cell lysate with concentrated PrP<sup>C</sup>. As demonstrated in our previous [21] and current studies, the abundance of PrP<sup>C</sup> is a critical factor to guarantee robust PrP<sup>Sc</sup> amplification.

Our data suggest that, by adjusting PrP<sup>C</sup> levels to wild type brain material, lysate of newly engineered and previously established animal cell lines can serve as an excellent substrate for PMCA, in which PrP<sup>C</sup> maintains its native states of subcellular localization, conformation, glycosylation, disulfide bridging, and glycosylphosphatidylinositol anchoring [40,42,53,54]. Since these post-translational modification states can affect the efficiency of PMCA [17,22], use of material that include PrP<sup>C</sup> in its native form localized in the lipid rafts might result in more efficient PMCA than a similar reaction with recombinant PrP that lacks native conformation and post-translational modifications.
Interestingly, our results demonstrated that HIV-1 Gag does not affect PrPSc conversion, suggesting that retroviral Gag functions in PrPSc propagation through the mechanism other than the PrP conversion process. Instead, it may play a role in the regulatory mechanisms for prion susceptibility and maintenance of prion infection at the cellular level as evidenced by Gag-enhanced release of mouse-adapted scrapie from cell cultures [55].

To date, the existence of the “auxiliary factors” for PrP conversion remains unknown. Recent PMCA studies showed that conversion of purified and recombinant PrPSc to infective PrPSc was successful in the absence of any cofactor [44]. In contrast, other lines of PMCA-based investigations suggest the presence of a cofactor for PrP conversion [14,23,24,45,56]. Some demonstrate enhanced PrP conversion by supplementation with undefined crude homogenate or partially purified fractions of cells and tissues [23,39,56], while others specify RNA [14] and plasminogen [24] for the cofactor activity. However, the tissue specific presence or utilization of this cofactor remains to be further investigated. A recent report proposed the existence of a brain-specific cofactor [57], whereas other studies indicated the presence of the cofactor is not limited to the brain. In fact, PrP conversion by PMCA is successful with spleen and muscle tissue homogenate [58] and PMCA with purified PrPSc is equally robust when supplemented with tissue homogenate of major wild type organs including the brain [23]. In agreement, our results showed that lysis of both neuronal and non-neuronal cells supported robust PMCA independent of the origin of the cell types. This suggests that the cofactor for PrP conversion is unlikely to be present exclusively in the brain or in neurons, if it exists.

Cell lysate-based PMCA may provide convenience in examining PrP conversion influenced by the PrPSc sequence variability. PrPSc of various species or with either mutations or polymorphisms can be expressed in the mammalian cell lines and the cell lysate can be used for PMCA without purification. This offers a practical alternative to using brain material of limited availability or purified and recombinant PrP that require laborious, time-consuming procedures for preparation.

In summary, lysis of cultured cells that express PrPSc is an excellent substrate source to amplify PrPSc in PMCA. Validation of cell lysate-based PMCA introduces a convenient model system.

Figure 2. PMCA using cell lysate of RK13MoPrP and RK13. (A) Amplification of protease-resistant PrPSc of RML prions. The level of PK-resistant PrPSc before (−) and after (+) PMCA was compared by Western blotting using monoclonal anti-PrP 6H4 antibody. Prion-sick (RML) and normal (NBH) brain homogenate were diluted 100–24,000 fold in cell lysate (CL) of RK13MoPrP and RK13. PMCA using wild type FVB/N brain homogenate (BH) was conducted as a control. (B) Serial PMCA with RK13MoPrP cell lysate as substrates. Initially, RML seeds were diluted 100–1,000,000 fold, and then the products of PMCA in each round were diluted 10 fold thereafter. The PK-resistant PMCA products were detected by Western blotting using monoclonal anti-PrP 6H4 (panel A) and 5C6 (panel B) antibodies.

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Figure 3. PrPSc amplification affected by PrPC abundance in cell lysate. PMCA was performed using undiluted and diluted (1:10 fold) RK13MoPrP cell lysate (CL). Both RML and NBH were used as seeds in dilutions of 100–24,000 fold. PK-treated pre- (-) and post-PMCA (+) samples were analyzed. Western blotting was performed using monoclonal anti-PrP 6H4 antibody. doi:10.1371/journal.pone.0018047.g003

Figure 4. Influence of Gag expression to PrPSc amplification. PMCA using RK13MoPrP and RK13MoPrP-gag cell lysate was conducted with (1,800–180,000 fold diluted) or without (no seeds) RML seeds. Cell lysate of RK13vector that lacks expression of both PrPC and Gag was used as a control. Pre- (-) and post-PMCA (+) samples were treated with PK and analyzed by and monoclonal anti-PrP 5C6 antibody was used for Western blotting. doi:10.1371/journal.pone.0018047.g004
that provides unlimited flexibility in functional analysis of PrP conversion associated with diverse PrP sequence variances.

**Materials and Methods**

**Ethics statement**

The experiments using animals were 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 the University of Kentucky (IACUC ID Number: 2006-0044). The procedures to establish new cell lines were carried out based on the protocol approved by the Institutional Biosafety Committee of the University of Kentucky (Registration Number: B10-795).

**Cell lines**

The cell lines used for the study are following: a rabbit kidney epithelial cell line RK13(ATCC, CCL37) [59]; a mouse neuroblastoma cell line, N2a (ATCC, CCL131) [48]; a scrapie-infected mouse brain cell line cured by pentosan sulfate, SMB-PS [49]; a mouse fibroblast cell line, NIH3T3 (ATCC, CRL1658) [51]; a mouse cerebellar cell line, CRBL [50]; a PrP knockout mouse hippocampal neuronal cell line, Hpl 3–4 [60]; two RK13 cell lines that either express mouse PrP C alone (RK13MoPrP) or coexpress both mouse PrP C and retroviral Gag protein (RK13MoPrP-gag); and RK13 cells that express Syrian hamster PrP C (RK13SHaPrP). RK13MoPrP and RK13SHaPrP were established by stable transfection of RK13 cells that lack expression of endogenous PrPC with the PrP open reading frames of each species cloned in the mammalian expression vector pIRESpuro3 (Clonetech, Palo Alto, CA) by the method described previously [61]. RK13vector was established by transfection with empty pIRESpuro3 plasmid. The stable transfectants were obtained by selection with 1 µg/ml puromycin. RK13MoPrP-gag was established by stably transfecting RK13MoPrP with pcDNA3-gag that harbors the gene for HIV-1 Gag precursor protein. RK13MoPrP-gag was selected with 1 mg/ml Geneticin.

**Figure 5. PMCA using lysates of a wide range of cell types.** Cell lysate of neuronal (N2a), prion-free brain mesenchymal (SMB-PS), mixed cerebellar neuronal and glial (CRBL) or fibroblast (NIH 3T3) cells was concentrated to include the PrP C level of wild brain homogenate. PMCA was performed by seeding with RML-sick (RML) and normal (NBH) brain homogenate. The seed dilution fold was 100–24,000. The PrP C level of PMCA before (−) and after (+) was compared. Monoclonal anti-PrP 6H4 antibody was used for Western blotting.

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sulfate (G418, Invitrogen, Carlsbad, CA), as previously described [47].

Cell culture

Culture of the cell lines were conducted as described elsewhere [50]. Briefly, cells were grown in Dulbecco’s Modified Eagle’s Medium (high glucose, Invitrogen) containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamax under saturated humidity and 5% CO₂ conditions at 37 °C.

Fluorescence microscopy

RK13MoPrP cells cultured onto 22 x 22 mm square glass cover slips were fixed with 4% paraformaldehyde and blocked with 10% goat serum (Invitrogen). For PrP staining, cells were incubated with 0.5 μg/ml monoclonal anti-PrP primary antibody, 6H4 (1:500 dilution) (Prionics, Zurich, Switzerland) and fluorescein-conjugated goat anti-mouse IgG secondary antibody (1:500 dilution) (Jackson ImmunoResearch Lab Inc, West Grove, PA) for 45 min each at room temperature. For GM1 staining, cell were

Figure 6. Comparison of ability of cell lysate to support PMCA. (A-C) The levels of PrPSc in pre- (–, dashed line with filled symbols) and post-PMCA (+, solid line with open symbols) samples shown in Figures 2, 3, and 4 were measured by densitometry and presented as relative % to that of the 1:100 diluted PrPSc seeds (n = 3 each). (D) Efficacy of PMCA supported by different PrPSc sources was presented as fold increase of the PrPSc level compared to the PrPSc level of seeds in each dilution. The data sets for transgenic mice that overexpress PrPSc in the brain (Tg4112) was obtained from the Western blots previously published [21].

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incubated with the Alexa Fluor 594-conjugated cholera toxin B subunit (1:50 dilution) (Invitrogen) for 45 min. Counterstaining of nuclei using 5 μg/ml Hoechst 33342 dye (Invitrogen) was performed simultaneously during incubation with the secondary antibody. The cover slips mounted on glass slides with 15 μl Mowiol mounting medium were immediately observed via a Leica AOBS TCS SP5 inverted laser scanning confocal microscope.

PMCA with cell lysate

PMCA was conducted by using cell derived material as the PrPSc source. Cultured cells in the 100 mm-diameter culture plates were washed twice with 10 ml phosphate buffered saline (PBS). The adhered cells were harvested in 1ml PBS using a cell scraper. Depending on the level of PrPSc in each cell line, cells in 5–15 plates were combined and centrifuged at 1,000 g for 5 min at 4°C. Pellet was washed with 10ml PBS once again before resuspended in 1 ml PMCA conversion buffer [PBS, pH 7.2, 150 mM NaCl, 1% Triton X-100, 4 mM EDTA, 1X CompleteMini protease inhibitors (Roche)]. Cell lysate was made by serially passing through hypodermic needles from 16 to 21 gauges. After centrifugation at 2,000xg for 5 min at 4°C, supernatant was used as the substrate for PMCA.

Brain homogenate (10% w/v) of terminally ill FVB mice inoculated with RML prions or Syrian hamsters inoculated with either the HY or DY TME agents was used as the source for PrPSc seeds. For PMCA, the seed was diluted as indicated (10^2–10^6 fold) in 0.1 ml cell lysate. Then, PMCA was performed as previously described [21]. Briefly, 94 cycles of amplification was conducted at 37°C with periodic 40 s sonication every 30 min using Misonix Model 3000 (Farmingdale, NY). For serial PMCA, the PMCA products from previous amplification rounds were diluted 10 fold in 0.1 ml of fresh cell lysate and subjected to repeated cycles of amplification as described above. Total four rounds of PMCA were carried out for the current study.

Western blot analysis

Western blotting for cell lysate to measure the expression level of PrPC was performed with samples not treated with PK. However, the pre- and post-PMCA samples were treated with PK (100 μg/ml) for 1 hr at 37°C, and then subjected to Western blotting. The procedure followed the protocol described in the previous publication [50]. Mouse and Syrian hamster PrP was recognized by incubation with monoclonal anti-PrP antibodies, 6H4 (Prionics, Zurich, Switzerland), D13 (Inpro, South San Francisco, CA), 3F4 (Signet Laboratory, Boston, MA), and 5C6 (raised against full length recombinant cervid PrP. G. Telling, unpublished data). Monoclonal anti-β-actin (ACTN05, Neomarker, Fremont, CA) and anti-HIV p24 (Chemicon, Temecula, CA) antibodies were
used to detect the expression of β-actin and Gap, respectively. The peroxidase conjugated goat anti-mouse IgG (Pierce, Rockford, IL) antibody was used as the secondary antibody. The signal was developed by using the ECL plus substrate (Amersham Pharmacia, Piscataway, NJ) and visualized by scanning with the Fuji Film FLA 5000 (Fuji Film, Edison, NJ).

Doc-It Image Analysis Software (UVI, Upland, CA) was used for densitometry analysis. Densitometry data were used to estimate the levels of PrPSc expressed in each cell line and PrPSc generated by PMCA. The level of newly generated PrPSc and efficiency of PMCA was calculated as described previously [21].

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Author Contributions

Conceived and designed the experiments: CR CEM JCB. Performed the experiments: CEM HEK JB VK YK. Analyzed the data: CR CEM HEK JB GCT JCB. Contributed reagents/materials/analysis tools: GCT JCB. Wrote the paper: CR CEM JCB.

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