Virology 382 (2008) 267–276

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

In vitro strain adaptation of CWD prions by serial protein misfolding cyclic amplification

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ARTICLE INFO

Article history:
Received 7 August 2008
Returned to author for revision
8 September 2008
Accepted 12 September 2008
Available online 25 October 2008

Keywords:
Prions
Chronic wasting disease
Protein misfolding cyclic amplification

ABSTRACT

We used serial protein misfolding cyclic amplification (sPMCA) to amplify the D10 strain of CWD prions in a linear relationship over two logs of D10 dilutions. The resultant PMCA-amplified D10 induced terminal TSE disease in CWD-susceptible Tg(cerPrP)1536 mice with a survival time approximately 80 days shorter than the original D10 inoculum, similar to that produced by *in vivo* sub-passage of D10 in Tg(cerPrP)1536 mice. Both *in vitro*-amplified and mouse-passaged D10 produced brain lesion profiles, glycoform ratios and conformational stabilities significantly different than those produced by the original D10 inoculum in Tg(cerPrP)1536 mice. These findings demonstrate that sPMCA can amplify and adapt prion strains *in vitro* as effectively and much more quickly than *in vivo* strain adaptation by mouse passage. Thus sPMCA may represent a powerful tool to assess prion strain adaptation and species barriers *in vitro*.

Introduction

According to the prion hypothesis, a proteinacious pathogen devoid of instructional nucleic acid initiates and propagates transmissible spongiform encephalopathies (TSEs), a group of invariably fatal, infectious neurological diseases (Prusiner, 1982) characterized by auto-conversion of the normal host cellular prion protein (PrP^{C}) into a misfolded, insoluble, proteinase K (PK)-resistant form (PrP^{RES}). Mounting biochemical and biological evidence supports the prion hypothesis (Büeler et al., 1993; Hope et al., 1986; Oesch et al., 1985; Prusiner, 1982). Prion infectivity correlates with PrP^{RES} in brain homogenates from animals afflicted with TSEs, including sheep scrapie, bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD, (Bolton et al., 1982; Browning et al., 2004; Collinge et al., 1995a; Hope et al., 1988; Race et al., 2002; Scott et al., 1999). More recently, synthetic prions made from truncated recombinant PrP have been shown to be infectious when inoculated into transgenic mice expressing the same isoform and in wild type mice upon subsequent passage (Legname et al., 2004). Additional evidence has come from several reports that demonstrate *in vitro* generation of infectious hamster prions using serial protein misfolding cyclic amplification (sPMCA), a highly efficient amplification method employing repeated cycles of incubation of prions with normal brain homogenate (NBH) as a source of PrP^{C} substrate to grow existing prion templates, and sonication to break the resulting large aggregates into smaller, more numerous prion templates (Castilla et al., 2005; Deleault et al., 2007; Murayama et al., 2007a; Weber et al., 2007). sPMCA of PrP^{RES} from mouse-adapted scrapie (PrP^{Sc}) and CWD prions (PrP^{CWD}) has recently been described (Kurt et al., 2007; Murayama et al., 2007b), although infectivity of these amplified materials was not assessed.

The unique etiology of mammalian prion diseases complicates characterization, identification, and even the definition of prion strains. Traditionally, prion strains have been typed according to host range, incubation time to terminal disease and neuroanatomic lesion profiles based on seminal work comparing human and animal TSEs (Gajdusek et al., 1966; Gibbs and Gajdusek, 1971; Hadlow, 1959; Taraboulos et al., 1992). Other criteria have been developed based on biochemical and biophysical properties of prions to investigate heritable structural differences among different prion strains, including size and extent of the PK-resistant core (Bessen and Marsh, et al.1994; Korth et al., 2003; Telling et al., 1996), glycoform ratio and conformational stability upon chemical or thermal denaturation (Collinge et al., 1996; Hill et al., 1997; Peretz et al., 2001, 1997; Safar et al., 1993, 1998; Scott et al., 2005). These parameters have proven useful for identification of prion strains with similar origins, host ranges and pathology and to predict transmission barriers to heterologous host species (Collinge and Rossor, 1996; Raymond et al., 2000; Will et al., 1996).

Experimental inoculation of animals represents the most reliable and accepted measure of efficiency of strain adaptation and transmission of prions into new hosts (Collinge et al., 1995a, 1995b; Hamir et al., 2005, 2006a, 2006b, 2003). However, these experiments often

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0042-6822/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2008.09.023

require extraordinarily long incubation periods, even by prion experimental standards, to fully assess strain adaptation and species barriers. Here we report efficient linear amplification of PrP^{CWD} by sPMCA resulting in *in vitro* generation of infectious CWD prions. Remarkably, we observed a drastic, nearly identical reduction in incubation time to terminal disease of CWD-susceptible Tg(cerPrP) 1536 mice inoculated with *in vitro*-amplified or mouse-passaged prions from the D10 isolate of CWD prions when compared to the original D10 inoculum. *In vitro*-amplified and *in vivo*-passaged D10 also shared similar neuropathological and biochemical characteristics that were significantly different than the original D10 strain, more closely resembling the RML strain of mouse-adapted scrapie prions. By all accepted parameters used to characterize prion strains that we examined, sPMCA adapted the D10 CWD strain as efficiently as passage in Tg(cerPrP)1536 mice, and represents a powerful, efficient tool for assessing strain adaptation and species barriers *in vitro*.

Results

Linear amplification of PrP^{CWD}

In vitro amplification of PK-resistant PrP (PrP^{RES}) from CWD prions using protein misfolding cyclic amplification (PMCA) has recently been reported (Kurt et al., 2007). We extend this work in several ways. We first tested whether NBH expressing heterologous PrP^{C} could be used to amplify PrP^{RES}. We performed PMCA of D10 and RML serially diluted into NBH from wild type FVB mice (Fig. 1A) and TgA20 mice expressing 4–5-fold more mouse PrP^{C} (moPrP^{C}, Fig. 1B). Both moPrP^{C} substrates supported amplification of scrapie PrP^{RES} (PrP^{Sc}), but not CWD PrP^{RES} (PrP^{CWD}). We then performed PMCA on D10 and RML samples serially diluted into Tg(cerPrP)1536 NBH expressing five-fold more cervid PrP^{C} (cerPrP) than FVB mice express moPrP^{C} (C). After PMCA we detected a 6×10^{6}-fold dilution of PrP^{CWD} (lane 8), whose band intensity matched that of the 9×10^{2}-fold dilution of unamplified D10 (lane 3), thus yielding an approximately 6666-fold increase in PrP^{CWD}. Tg(cerPrP)1536 NBH failed to support PrP^{Sc} amplification. Quantitative analyses of band intensities from three experiments demonstrated a consistent, rapid decrease in band intensity of unamplified PrP^{CWD} dilutions, with complete loss of signal at 10^{-3} dilutions (D). Plotting −fold amplification as a function of −fold dilution of D10 on a log–log scale reveals a linear relationship between 10^{3} and 2×10^{5}-fold dilution of D10 (E).

sPMCA or mouse passage of D10 shortens mean incubation time to terminal prion disease

To determine whether amplified PrP^{CWD} is infectious, we used serial PMCA (sPMCA) to generate PrP^{CWD} *in vitro*. From our quantification data, repeated 10^{-3} dilution and amplification should maintain sPMCA in the linear range of PrP^{CWD} amplification (Fig. 1E). Beginning with a 10^{-3} D10 dilution, we achieved a ten-fold amplification efficiency after one PMCA round, producing PrP^{CWD} equivalent to a 10^{-2} dilution. Re-diluting this material 10^{3}-fold produced a 10^{-5} dilution equivalent. We then amplified this material approximately 10^{3}-fold, again producing a 10^{-2} equivalent dilution. We continued this sPMCA strategy for six more rounds, oscillating between 10^{-5} and 10^{-2} equivalent dilutions upon re-dilution and PMCA to arrive at an overall D10 dilution of 10^{-24}, a point at which the original PrP^{RES} inoculum has been estimated to be lost, and only amplified PrP^{RES} is present in the sample (Castilla et al., 2005). Western blot analysis demonstrated the maintenance of a strong PrP^{RES} signal in the amplified product throughout sPMCA, whereas unamplified samples were undetectable (Fig. 2A). Densitometric analysis revealed similar band intensities for PK-digested D10, amplified 10^{-24} D10 and mouse-passaged D10, with RML producing approximately half the intensity of the other three inocula (Fig. 2B). We therefore inoculated Tg(cerPrP)1536 mice intracerebrally with 30 µl of each D10-derived inoculum diluted 1:10 in sterile 320 mM sucrose supplemented with antibiotics in PBS. All mice inoculated with amplified D10 contracted neurologic disease consistent with TSE with a mean incubation time to terminal illness of 169±4 days post inoculation (DPI), while all mice inoculated with unamplified D10 are currently asymptomatic at >500 DPI (Table 1). Surprisingly, Tg(cerPrP) 1536 mice inoculated with the original D10 strain contracted disease 82 days later (251±3 DPI) than amplified D10-inoculated mice. Inoculation of Tg(cerPrP)1536 mice with mouse-passaged D10 (D10

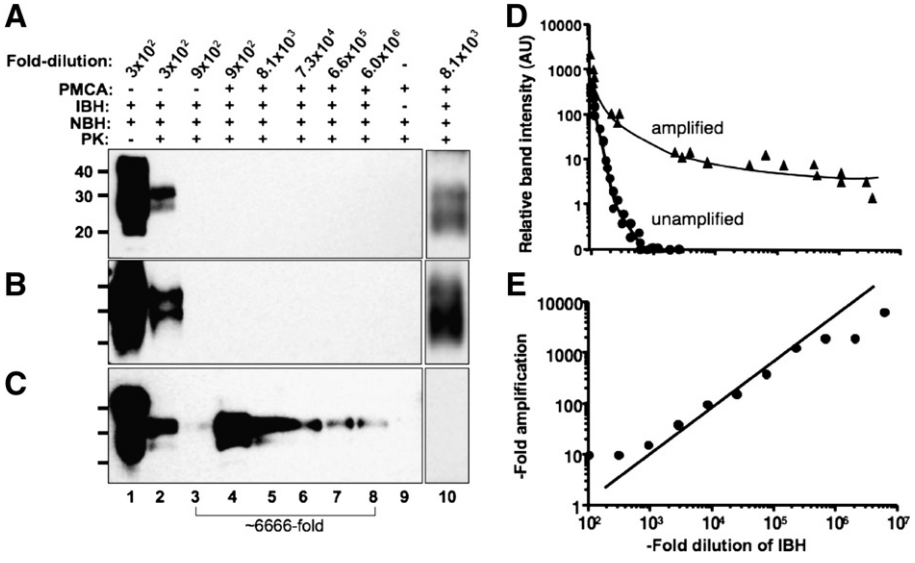

Fig. 1. Protein misfolding cyclic amplification of chronic wasting disease prions. Three-fold serial dilutions of infected brain homogenate from the D10 isolate of CWD prions (D10, lanes 1–9) or Rocky Mountain lab strain of mouse-adapted scrapie prions (RML, lane 10) diluted $1:3\times10^{2}$ into normal brain homogenate (NBH) were either snap-frozen (lanes 1–3) or subjected to 96 cycles of PMCA (lanes 4–10). Proteinase K (PK) digestion and western blotting of samples reveal that wild type NBH (A) or TgA20 NBH (B) supported PrP^{Sc} (lane 10), but not PrP^{CWD} (lanes 4–9) amplification, whereas Tg(cerPrP)1536 NBH (C) efficiently amplified PrP^{CWD} (lanes 4–9), but not PrP^{Sc} (lane 10), resulting in approximately 6666-fold PrP^{CWD} amplification after one PMCA round (compare lanes 3 to 8). Molecular weight markers are shown in kDa to the left of the blots. (D) Quantification of band intensities demonstrates a dramatic decrease in signal intensity upon dilution of D10 without PMCA, and sustained intensity to 6×10^{6}-fold dilution with PMCA. (E) Plotting −fold amplification as a function of D10 dilution reveals a linear relationship over two logs of D10 dilutions. Data are representative of at least three independent experiments.